Synthesis of novel benzotriazole substrates suitable for screening hydrolytic enzymes

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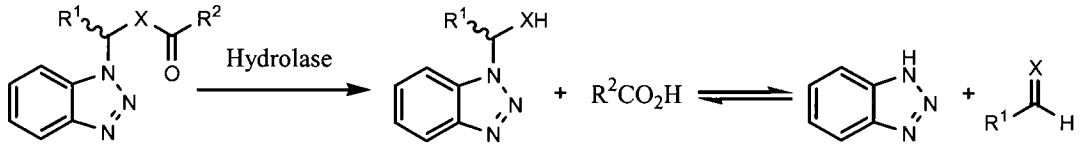
Abstract

1-(benzotriazol-1-yl) alkyl esters (**94a-e, 105** and **112**) and amides (**117-118**) have been investigated as substrates suitable for screening hydrolytic enzymes. Following enzymatic hydrolysis, in aqueous media, the reaction products fragment to release benzotriazole – **Scheme 1**.



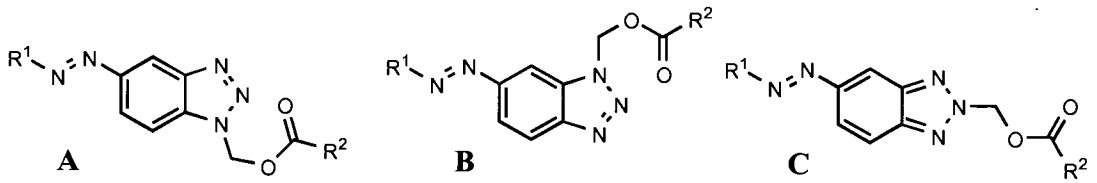
94 a-e, 105, 112 (X=O)
117-118 (X=NH)

R^1 = H, CH_3, CH_2CH_3, $CH(CH_3)_2$, *p*-$NO_2C_6H_4$
R^2= alkyl, benzyl, $PhCH_2OCONHCH(CH_2Ph)$

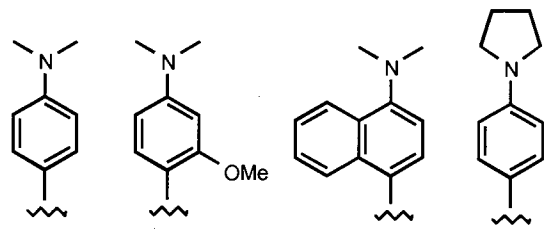
Further hydrolysis

Scheme 1: *Enzymatic hydrolysis of 1-(benzotriazol-1-yl)alkyl esters and amides*

The synthesis of novel surface enhanced resonance raman spectroscopy (SERRS) inactive mono-azo benzotriazole dyes (*S*) /(*R*) **141-144 A**, **B** and **C** and their application to screening enzymatic reactions is described – **Figure 1**.



Where R^1 =



141 **142** **143** **144**

Where R^2 = (*R* or *S*) - $CH_2CH(Ph)CH_3$

Figure 1: *SERRS inactive mono-azo benzotriazole dyes.*

Abbreviations

anh.:	anhydrous
br:	broad
Bt:	benzotriazole
CE:	capillary electrophoresis
CBz	carboxybenzyl
$CDCl_3$	deuteriochloroform
CH_3Cl:	chloroform
CH_3CN:	acetonitrile
d:	doublet
DEAD	diethylazodicarboxylate
DCC	1,3-dicyclohexylcarbodiimide
DCM:	dichloromethane
DIPEA:	di-*iso*-propylamine
DMAP:	*N'N'*-dimethyl-4-aminopyridine
DMF:	dimethyl formamide
DMSO:	dimethyl sulphoxide
DBU:	1,8-diazobicyclo[5.4.0]undecane-7
e.e.:	enantiomeric excess
EDCI:	1-(3-dimethylaminopropyl)-ethylcarbodiimide
ELISA:	enzyme linked immunosorbant assay
ESi:	electrospray ionisation
Et:	ethyl
Et_3N:	triethylamine
Et_2O:	diethyl ether
EtOAc:	ethyl acetate
EtOH:	ethanol
FAB:	fast atom bombardment
GC:	gas chromatography
HOBt:	1-hydroxybenzotriazole
HPLC:	high performance liquid chromatography
Hz:	hertz
IPA:	*iso*-propyl alcohol
m:	multiplet
Me:	methyl
Mp:	melting point
MeOH:	methanol
NMR:	nuclear magnetic resonance
nOe:	nuclear overhauser effect
ph:	phenyl
ppm:	parts per million

q:	quartet
s:	singlet
SERRS:	surface enhanced resonance raman spectrometry
t:	triplet
TFA:	trifluoroacetic acid
THF:	tetrahydrofuran
TLC:	thin layer chromatography

1. Introduction

1.0 Opening Remarks

Biocatalysis is now widely recognized as a practical alternative to traditional (non-enzymatic) organic synthesis and is increasingly implemented to solve industrially relevant problems[1]. One area, which has arguably received the most attention, is the application of hydrolytic enzymes to the preparation of enantiopure organic compounds. The importance of which, is perhaps best reflected in the pharmaceutical industry where the Food and Drug administration (FDA) require all newly registered drugs to be produced and characterised in enantiomerically pure form. This requirement has sound reasoning; since biological activity is often restricted to one of the two enantiomers and, in extreme cases, the 'wrong' enantiomer may exhibit undesirable side effects. Enzymes, in particular esterases and lipases, have been widely applied to the preparation of pure enantiomers from racemic mixtures, prochiral or *meso* compounds, or diastereomeric mixtures. This resolution can be achieved by hydrolysis in aqueous media or by the reverse reaction, transesterification/esterification, in organic solvents.

Many of the synthetic problems encountered can be solved, by choosing the correct enzyme and simply adding it to the reaction much like any other chemical reagent. Indeed, for some enzymes, simple rules exist to help predict which enantiomer will react quickest[2]. Unfortunately, most enzymes have evolved to contribute to the survival of an organism and will not perform well under the conditions required by the process chemist. However, several techniques have been developed to circumvent this: *e.g.* new enzymes can be identified by screening from a range of sources[3], the properties of existing enzymes can be manipulated by choosing the appropriate reaction conditions (medium engineering)[4] or mode of implementation (*e.g.* immobilization, cross-linking *etc*[5, 6]). Ultimately, many problems are best solved by engineering the biocatalyst itself, either by: directed evolution (combinatorial approach)[7-15], site-directed mutagenesis (rational approach)[16-18] or a combination of both techniques to generate libraries of

'mutant' enzymes which may comply with the chemists wishes. A schematic of the numerous manipulation options available is shown - **Figure 1**.

Unfortunately, the testing of a number of commercially available enzymes or mutated enzyme libraries presents a significant scientific challenge in itself, primarily due to the time and labour involved. This is in part due to the vast number of potential 'mutants' which can be generated by directed evolution and in the length of time required to analyse the enzymatic reaction by traditional techniques *e.g.* NMR, GC and HPLC. Scientific progress to circumvent this, has led to the development of a range of new high-throughput techniques to determine if the mutated enzymes have the desired property.[19-22]

In this thesis, the focus shall primarily be on the development of novel substrates suitable for screening lipolytic enzymes, although reference to other hydrolytic enzymes and enzyme classes will be made.

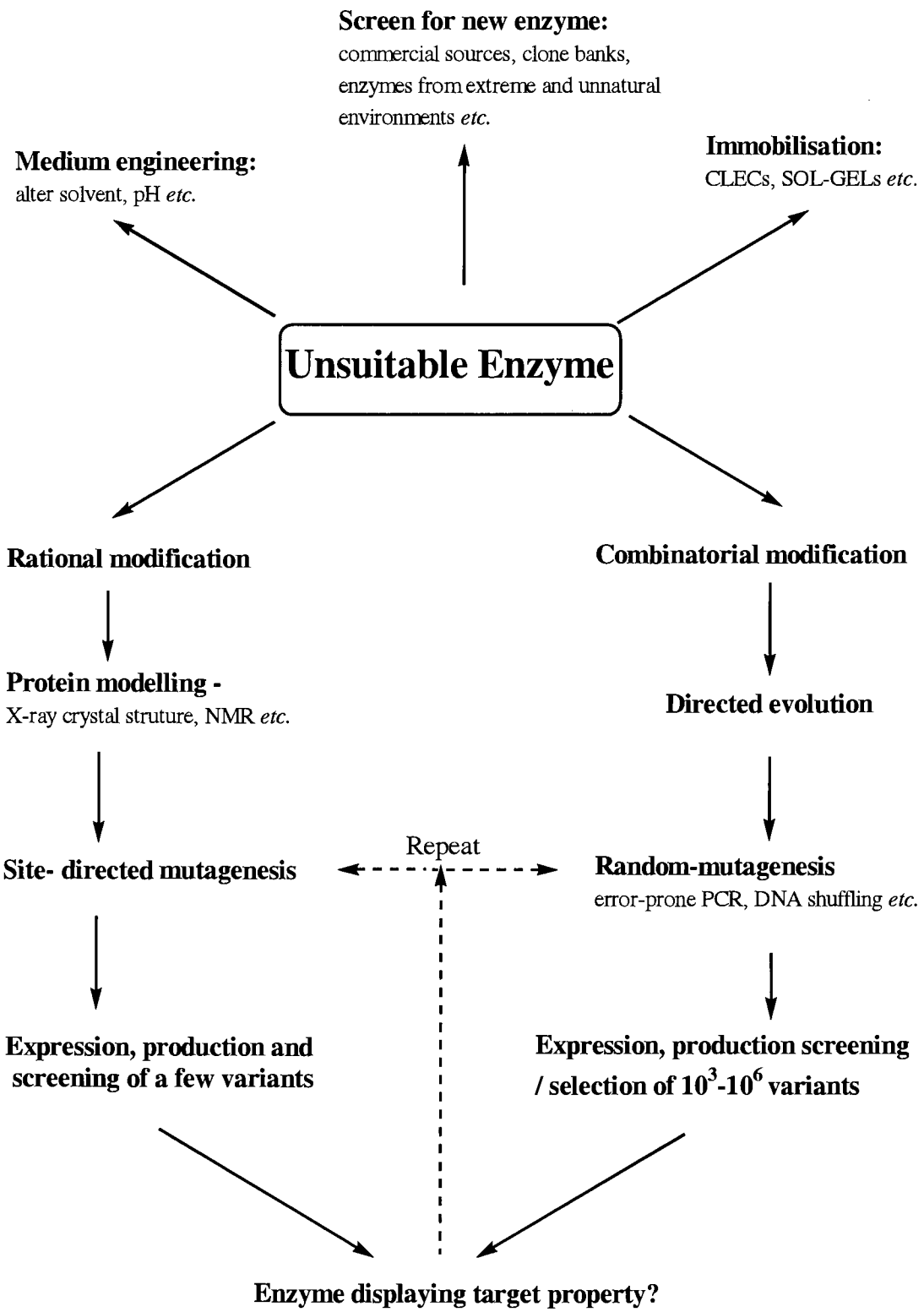
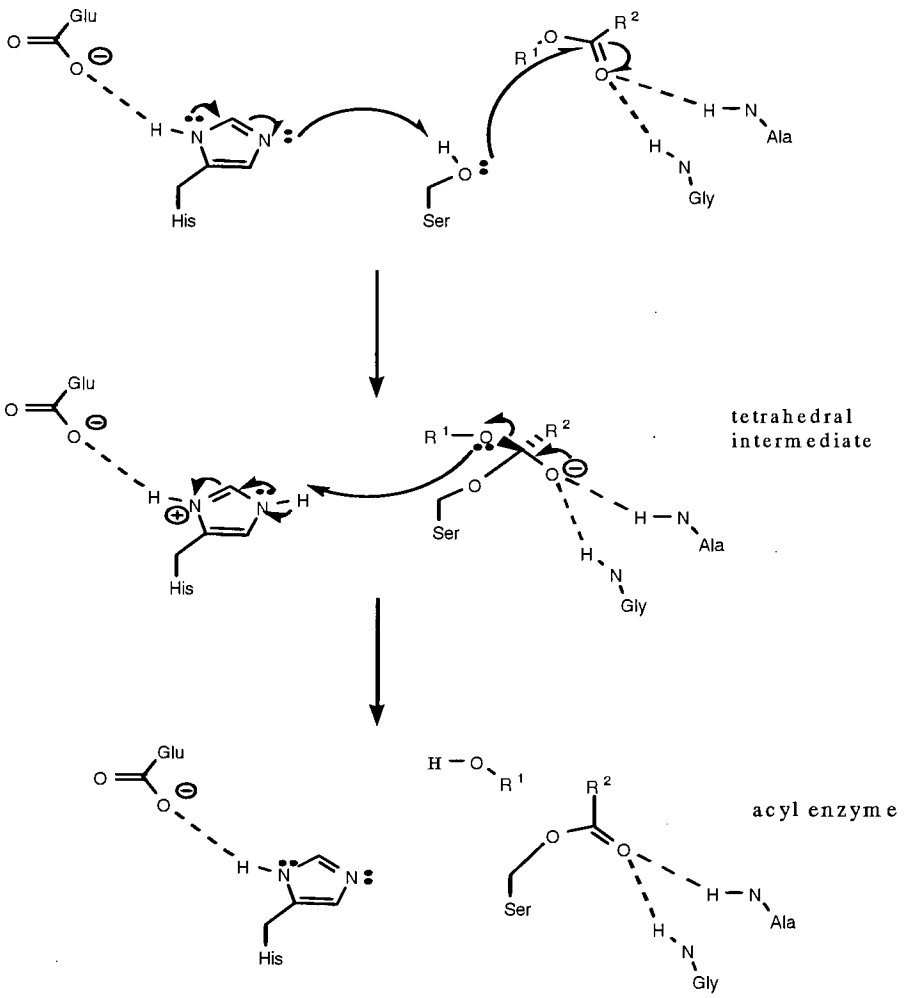


Figure 2: *Solutions available to solve biocatalytic problems*

1.1 Lipolytic enzymes

1.1.1 Role and action of lipases and esterases.

Lipases (triacylglycerol ester hydrolase's, EC 3.1.1.3) and carboxylesterases (EC 3.1.1.1) are enzymes widely distributed among animals, plants and micro-organisms. These two classes of enzymes catalyse the hydrolysis and synthesis of water insoluble long chain triglycerides and small partially water soluble esters, respectively. The mechanism of activity is the same for both classes and is based on a 'catalytic triad', made up of nucleophilic serine, histidine and aspartame/glutamate.[23] This 'catalytic triad' is similar to that found in serine proteases - **Scheme 2.**



Scheme 2: *Catalytic mechanism of lipases*

In contrast to esterases, lipases do not obey normal Michaelis-Menten kinetics displaying little or no catalytic activity at low substrate concentrations. Instead, a sharp increase in hydrolytic activity occurs when the triglyceride substrate concentration is increased beyond its critical micellular concentration to form an emulsion. This phenomenon was termed 'interfacial activation'[24] and the effect is displayed - **Figure 3**.

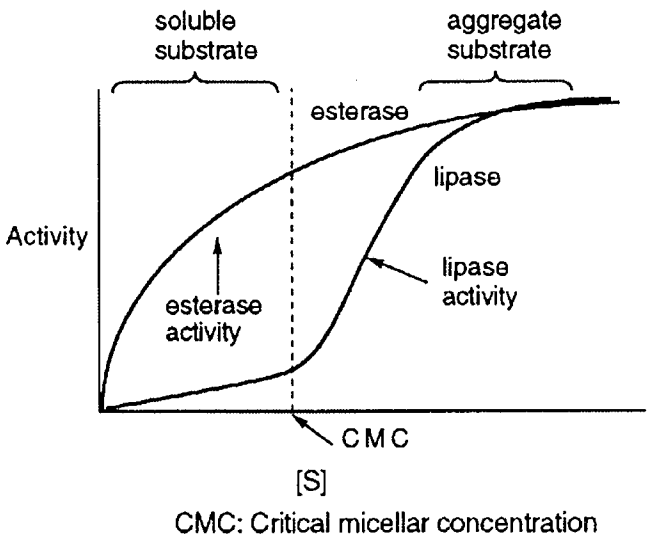
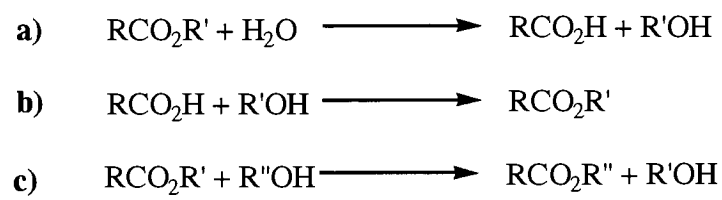


Figure 3: *Interfacial activation of lipases*[2]

Most lipases also contain a 'lid',[25-26] which is a surface loop of the protein covering the active site of the enzyme. The 'lid' moves away on contact with the interface thus allowing the substrate access to the active site. However, not all lipases have a lid present or show interfacial activation.[23]

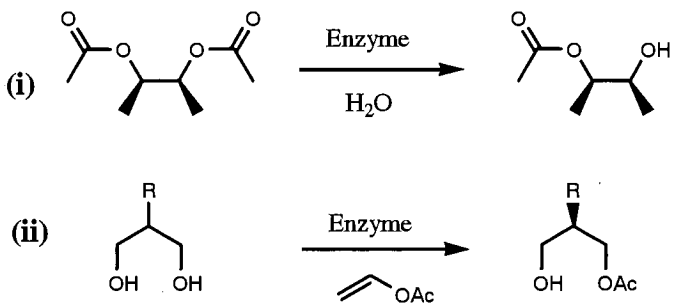
1.1.2 The use of lypolytic enzymes in organic synthesis

Both lipases and esterases have been widely used in synthetic organic chemistry,[1, 2, 27, 28] catalyzing the chemio-, regio- and/or stereoselective hydrolysis (**a**) of carboxylic acid esters in aqueous media or the reverse reaction *i.e.* esterification (**b**) or transesterification (**c**) in organic solvents - **Scheme 3**.

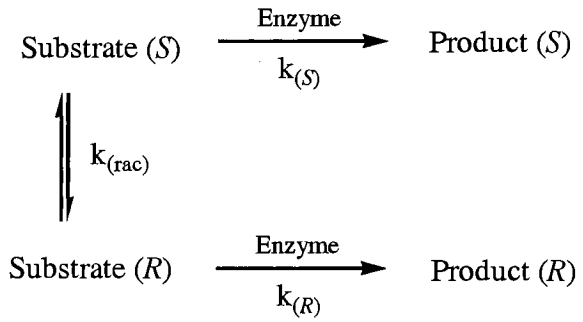


Scheme 3: *Reactions catalysed by lipases/esterases*

In any kinetic resolution the enzyme only utilises one enantiomer of the racemic starting material, limiting the maximum yield to 50%. In the majority of cases, this theoretical 50% is never achieved because the reactions are stopped at low conversion (20-30%) if optically pure product is required or 70-80% conversion if the substrate is required. The yield can be improved through a variety of techniques, which include: the desymmetrisation of *meso* or prochiral substrates[29-31] (**Scheme 4**) or dynamic kinetic resolution. Dynamic kinetic resolution comprises a kinetic resolution with in-situ substrate racemization[32, 33] - **Scheme 5**. For dynamic kinetic resolution to be effective two criteria must be fulfilled; firstly the rate of racemisation $k_{(rac)}$ must be greater than (or at least equal to) the rate of the enzymatic reaction $k_{(S)}$ for the faster reaction, and the kinetic resolution must be selective *i.e.* $k_{(S)} >> k_{(R)}$. A number of suitable racemisation catalysts have been reported, including organic bases,[34] transition metal catalysts containing rhodium[35, 36] and palladium on charcoal.[37]



Scheme 4: *Enzymatic resolution of* (**i**) *meso or* (**ii**) *prochiral compounds.*



Scheme 5: *Dynamic kinetic resolution*

In addition, lipases and esterases have also been pioneered[38] as reagents for selective removal of protecting groups from a range of polyfunctional compounds *e.g.* peptides and carbohydrates. This method of de-protection is particularly attractive with sensitive substrates, because enzymes generally operate under mild conditions.

In all these transformations manipulation of the reaction medium – termed medium engineering, can dramatically alter the selectivity of the enzyme. Some factors which can be manipulated include pH, organic solvent,[39] water activity, addition of metal ions,[40] surfactants,[41] enzyme memory[42] and substrate concentration. These concepts have been reviewed recently.[4, 43] However, as mentioned previously, sometimes an enzyme displaying the appropriate function cannot be found either because the function was never developed – due to evolutionary pressure or an appropriate enzyme has not been found despite a comprehensive screening procedure. In either case, a range of modification techniques, both chemical and genetic, can be applied to generate an appropriate biocatalyst.

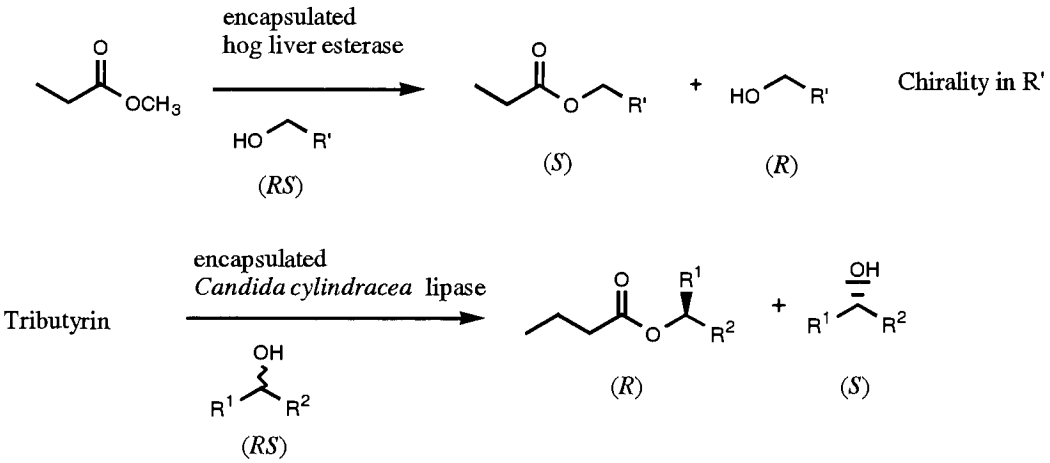
1.2 Modification of lipolytic enzymes

1.2.1 Chemical modification

Chemical modification processes have been extremely successful at improving the activity and enantioselectivity of enzymes, particularly in the application of lipases/esterases in organic solvents. One of the main drawbacks involved in the use of

enzymes in organic solvents is that they exhibit lower catalytic activity, primarily due to denaturation.

In 1984, Klibanov[44] encapsulated hog liver esterase and lipase from *Candida cylindracea* inside Sepharose or Chromosorb, respectively. The porous beads were suspended in organic solvents and the encapsulated enzymes exhibited high transesterification activity and stereoselectivity towards primary or secondary alcohols - **Scheme 6**. In these studies the organic solvent acted as both the reaction medium and the enzyme substrate.



Scheme 6: *Application of enzymes in organic solvents*

Following on from these initial experiments, a range of novel methods of chemical modification have been developed and reported. These include the immobilization of enzymes onto solid supports *e.g.* Lipozyme® (*Rhizomucor miehei* lipase immobilised on an anionic exchange resin), Novozyme® (*Candida antarctica* lipase B immobilised on acrylic resin) and more recently the introduction of cross-linked enzyme crystals, CLECs.[45] CLECs are prepared by cross-linking the enzyme with glyceraldehydes to provide biocatalysts with much increased stability and activity in organic media. Further developments include the pre-treatment of enzymes with organic

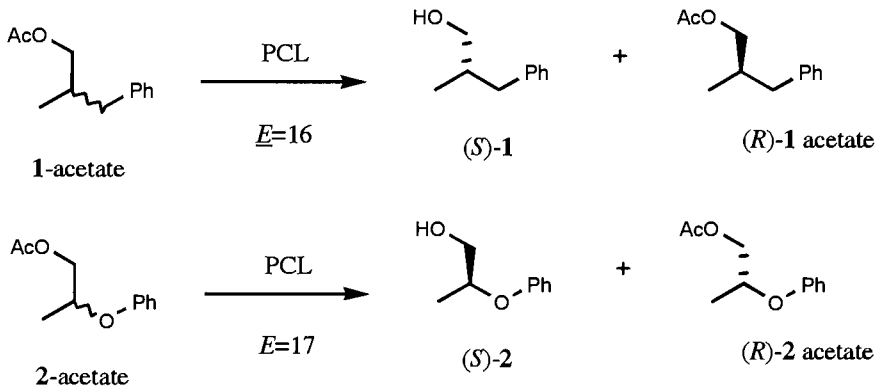
solvents,[46] entrapment in sol-gel matrices[47] and various other membranes. These chemical modifications have been discussed in a recent informative review article.[5]

If altering the mode of implementation of an enzyme is unsuccessful (in improving the enantioselectivity) then rational chemical modification of the primary structure of the biocatalyst may prove fruitful. The lipase from *Pseudomonas cepacia* has been successfully modified[48] by chemical modification. This example and other examples of rational design will be discussed further - **Section 1.2.2.**

1.2.2 Rational design of enzymes

To embark on rational design of an enzyme the relationship between protein sequence and function must be known. This relationship requires a detailed understanding of the three-dimensional structure and mode of action of the biocatalyst. For the vast majority of enzymes this information is unavailable and even if the target enzyme is well characterized, the molecular basis for the desired function may not be. Nevertheless, the introduction of computer modelling and the exponential growth of databases of protein structure and sequences have facilitated elegant advances in the rational design of enzymes.[49]

Kazlauskas *et al.*[48] used a combination of molecular modelling of transition state analogues and kinetic measurements to identify the molecular basis of the enantioselectivity of a lipase from *Pseudomonas cepacia* (PCL). The enantioselectivity of PCL towards the acetates of 2-methyl-3-phenyl-1-propanol **1** and 2-phenoxy-1-propanol **2** was studied - **Scheme 7**. Although PCL favours the (*S*)-enantiomer of both substrates, the (*S*)-enantiomers have different shapes due to changes in substituent priority.

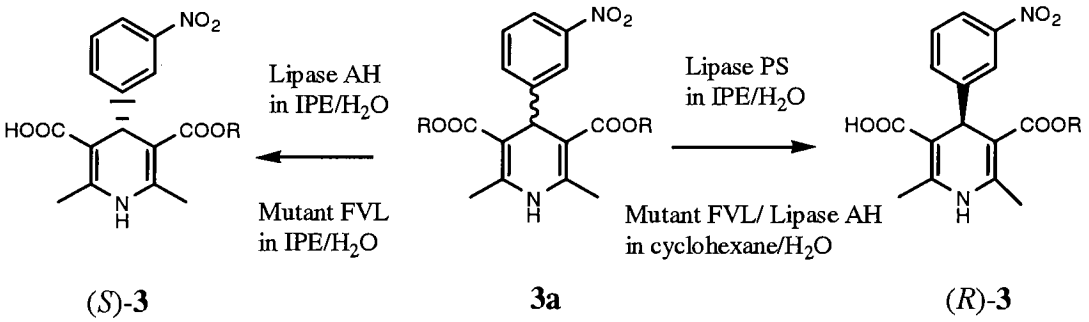


Scheme 7: *Enantioselectivity of PCL towards primary alcohols* **1** *and* **2**

The enantioselectivity towards **1**-acetate was due to binding of the methyl group (at the stereocentre) for the fast-reacting enantiomer but not the slow-reacting enantiomer. In contrast, the enantioselectivity towards **2**-acetate was due to an extra hydrogen bond between the phenoxy oxygen of the substrate with the phenolic OH of Tyr 29. Acetylation of Tyr 29 eliminated the hydrogen bonding toward **2**-acetate and resulted in a decrease in *E* from 16 to 8. Nitration with tetranitromethane, which increases the hydrogen bond strength of Tyr 29, increased the *E* value from 16 to 36 for **2**-acetate. The same research group have applied similar molecular molecular modeling techniques to rationalise the selectivity of *chromobacterium viscosum* lipase[50] and chymotrypsin.[51]

Inversion of enantioselectivity of lipase PS from *Pseudomonas cepacia,* by site-directed mutagenesis, was reported by Hirose and co-workers[17] in 1995. Comparison of the sequences of lipase AH (*Pseudomonas sp.*) and lipase PS indicated that they differed in only 16 amino acids but there enantioselectivity towards 1,4 dihydropyridines **3a** was complimentary *i.e.* lipase PS gave (*R*)-**3** while lipase AH gave (*S*)-**3** in IPE/H_2O - **Scheme 8**. Site-directed mutagenesis of lipase PS was performed, replacing Val^{266}, Leu^{287} and Phe^{221} for Leu, Ile and Leu respectively. The modified lipase PS (mutant FVL) now gave (*S*)-**3** (54%, >99.0% e.e. where R=$CH_2OCOC_2H_5$) in IPE/H_2O, a complete reversal of enantioselectivity.

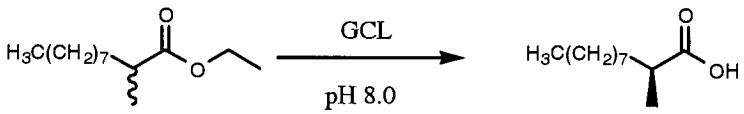
In addition, mutant FVL also showed reversal of enantioselectivity when the reaction solvent was changed from water-saturated IPE (product (*S*)-**3**) to water saturated cyclohexane (product (*R*)-**3**). An identical solvent effect had previously been observed for lipase AH.



(*S*)-**3** **3a** (*R*)-**3**

Scheme 8: *Reversal of enantioselectivity by site-directed mutagenesis*

In a more recent study, Holmquist and Berglund[52] utilised rational recombination and site-directed mutagenesis to modify the lipase from *Geotrichum candidum*. *Geotrichum candidum* produces two lipase isozymes (GCL I and II) which display different abilities to discriminate between the two enantiomers of ethyl 2-methyldecanote **4** - **Scheme 9**. By swapping homologous regions between the two isozymes, a hybrid enzyme (I 348-II-406-I) with improved enatioselectivity over the two wild types was created. In addition, site-directed mutagenesis of two residues (I Ile357Ala/Leu358Phe) gave a mutant enzyme with similar activity to wild type I but an increased *E* value from 32 to 54. These mutations were found to be located at the entrance to a tunnel, at the bottom of which lies the enzyme's active site. The presence of a tunnel was also found to be important in determining the selectivity of *Candida rugosa* lipase.[4, 53]



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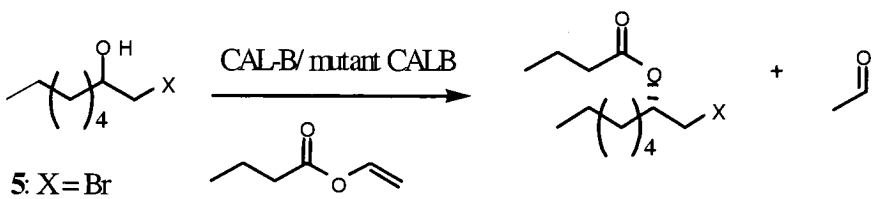
Wild type I: 89.9% e.e. (*E*=32)
Wild type II: 79.8% e.e. (*E*=10)
I-348-II-406-I: 95.4% e.e. (*E*=54)
I Ile357Ala/Leu358Phe: 95.5% e.e. (*E*=54)

Scheme 9: *Rational recombination and site-directed mutagenesis of GCL I and II*

Candida antarctica lipase B (CAL-B) displays poor enatioselectivity towards 1-halo-2-octanols **5** and **6** which is in sharp contrast to its high enantioselectivity towards 3-nonanol **7** - **Scheme 10**.[54 and refs therein] To investigate this phenomenon, molecular modelling of *Candida antarctica* lipase B (CAL-B) was used to 'virtually' mutate the lipase. The poor selectivity towards **5** and **6** was attributed to unfavourable interactions between the fast reacting (*S*)-enantiomer and a region situated at the bottom of the active site (termed the stereoselectivity pocket). These unfavourable interactions were 'virtually' removed, the mutants modelled, and then selected variants were generated by site-directed mutagenesis. The mutation Ser47Ala effectively doubled the enantiomeric ratio (at 60% specific activity) observed for **5** and **6**, while the mutation Trp104His annihilated the enantioselectivity towards **5** and **6** - **Table 1**. These results were attributed to changes in electrostatic potential and an increased volume of the stereoselectivity pocket, respectively.



5: X=Br
6: X=Cl
7: X=CH_3

Scheme 10: *Esterification of 1-halo-octanols using CAL-B*

CAL-B	*E*			Activity
	5	**6**	**7**	(tributyrin) [s-1]
Wild type	6.5 +/- 0.4	14 +/- 2	200	253 +/- 12
Ser47Ala	12.4 +/- 0.4	28 +/- 3	-	144 +/- 14
Trp104His	1.7 +/- 0.1	2.0 +/- 0.1	-	102 +/- 13

Table 1: *Enantiomeric ratios and activity for CALB mutants towards* **5**, **6** *and* **7**

Further CAL-B mutants were prepared by Patkar and co workers[55]. These studies focussed on modifications to the consensus sequence. Thr-X-Ser-X-Gly was modified to Gly-X-Ser-X-Gly the latter of which is the more usual sequence present in lipases. A T103G mutant showed increased thermostability but only half the specific activity. The specificity and enantioselectivity towards the acylation of a range of secondary alcohols remained unchanged. A W104H mutant displayed dramatically changed properties: with altered substrate specificity but lower thermostability, specific activity and enantioselectivity. The properties of the W104H mutant highlight the difficulty in predicting which amino acids are important for which function.

Again using CAL-B, the first example of an enantioselective hydrolase by engineered substrate-assisted catalysis (SAC) was reported.[56] The premise of this research was that the hydroxyl group of the substrate, in this case 2 or 3-hydroxy esters, **8** and **9**, could substitute for an active site residue. The active site residue had previously been removed by site-directed mutagenesis - **Figure 4**. CAL-B consists of the standard Ser-His-Asp catalytic triad and the transition state of the catalysed ester hydrolysis is an oxyanion. This oxyanion is stabilized by hydrogen bonding with two backbone amide linkages and the hydroxyl group of Thr 40. Two mutants Thr40Val and Thr40Ala were created and both showed 3 orders of magnitude lower k_{cat}/K_m values for ethyl propanoate and ethyl butanoate with respect to the wild type, thus indicating the importance of Thr40. The wild type lipase showed very low substrate specificity for either enantiomer of ethyl 2-hydroxypropanoate **8** compared to ethyl propanoate. In contrast, the mutants showed improved (*S*)-2-hydroxypropanoate/propanoate specificity indicating that the hydroxyl group with (*S*)-configuration is oriented to allow for

improved transition-state stabilisation. As expected, the mutants were unable to perform substrate-assisted catalysis with the (*R*)-enantiomer of **8**. The wild type lipase showed very low enantioselectivity toward **8** (E_S=1.6), whereas the Thr40Ala and Thr40Val mutants gave much improved values of E_S=9.8 and E_S=22, respectively. An increase in (*S*) specificity was also determined with ethyl 3-hydroxybutanoate **9** where the mutants displayed lowered E_R values (from E_R=31 in the wild type to E_R=7.9 and E_R=2.0 in Thr40Ala and Thr40Val, respectively. The significance of this development is clear when we consider that a large range of enzymes including subtilisin, papain and some lipases contain side-chain contributions to oxy-anion stabilization and could potentially be mutated in a similar manner.

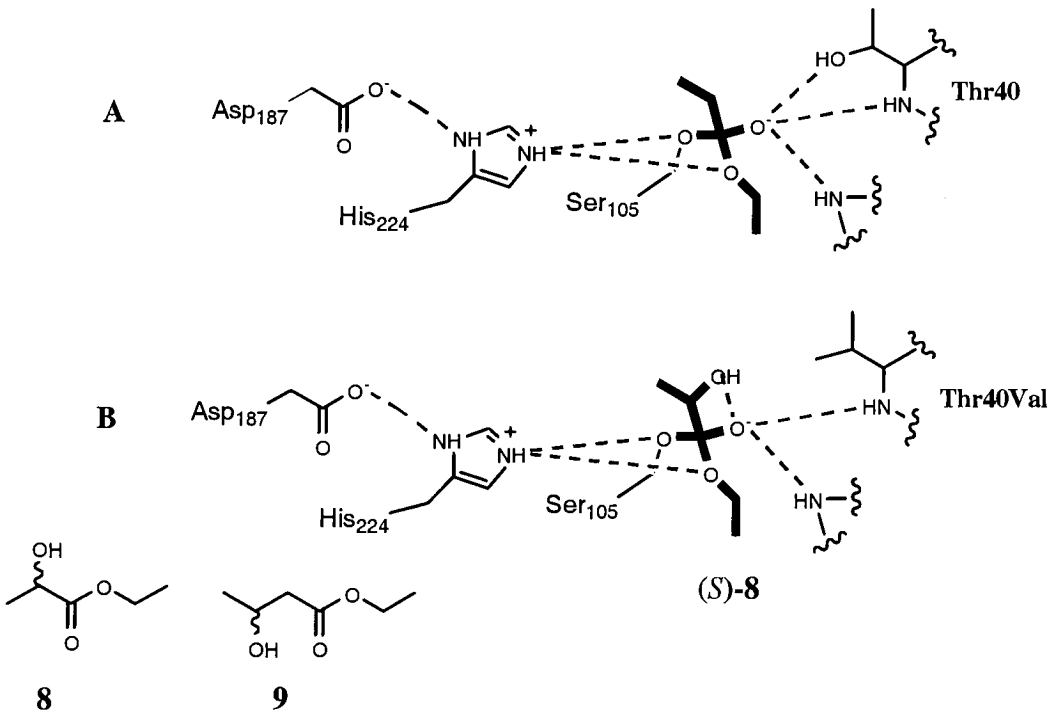


Figure 4: **A**: *wild type enzyme,* **B**: *Thr40Val mutant displaying substrate assisted catalysis.*

Recent reviews of lipase protein engineering have been published containing details of some of the variations which have been addressed[57] and the production of commercially important microbial lipases.[58]

1.2.3 Combinatorial design of enzymes

One distinct advantage of combinatorial (or directed) evolution of biocatalysts over rational design, is that the former requires no prior knowledge of the three dimensional structure of the catalyst. The former can be achieved either in an iterative fashion by the accumulation of point mutations (*e.g.* by low-rate error prone PCR) over a series of generations or by recombination of related genes (DNA shuffling[12, 13]). The key steps in a directed evolution process are shown - **Figure 5.** This technique has been applied to manipulate the properties of several different enzymes including the enantioselectivity and activity of lipases and esterases.

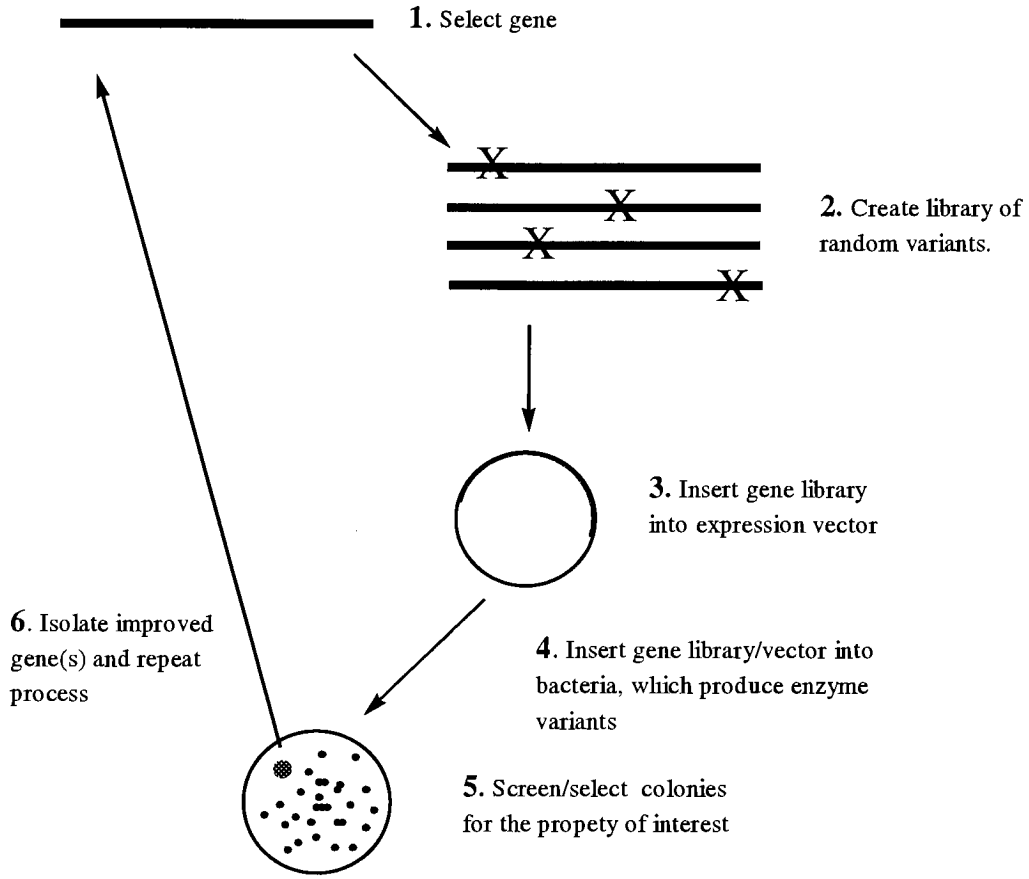
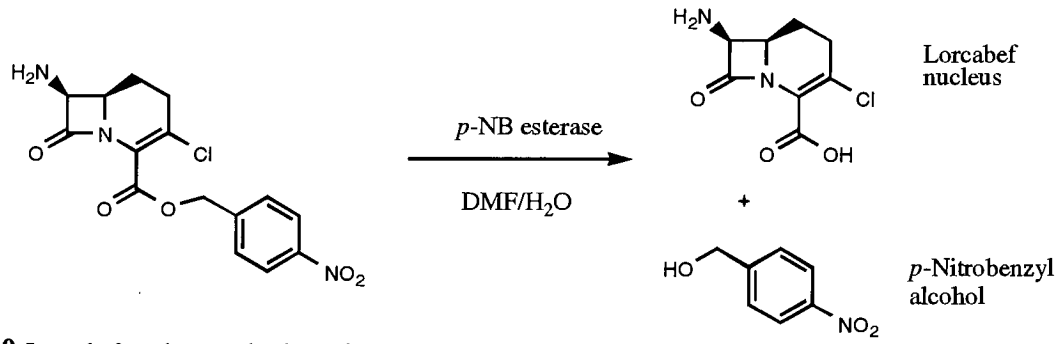


Figure 5: *Key steps in a typical directed enzyme evolution experiment*

Research in the Arnold[10] laboratories has successfully directed the evolution of Subtilisin E to enhance its activity in dimethylformamide. In 1996, Arnold and Moore[9] directed the evolution of a *para*-nitrobenzyl esterase (*p*NB) from *Bacillus subtilis* to enhance the enzymes activity towards the deprotection of the antibiotic Lorcabef nucleus *p*-nitrobenzyl ester **10** - **Scheme 11**. **10** is very insoluble in aqueous media, so the mutant enzyme must display high catalytic activity in aqueous DMF.



10-Lorcabef nucleus-*p*-nitrobenzylester

Scheme 11: *Deprotection of Lorcabef nucleus-p-nitrobenzyl ester*

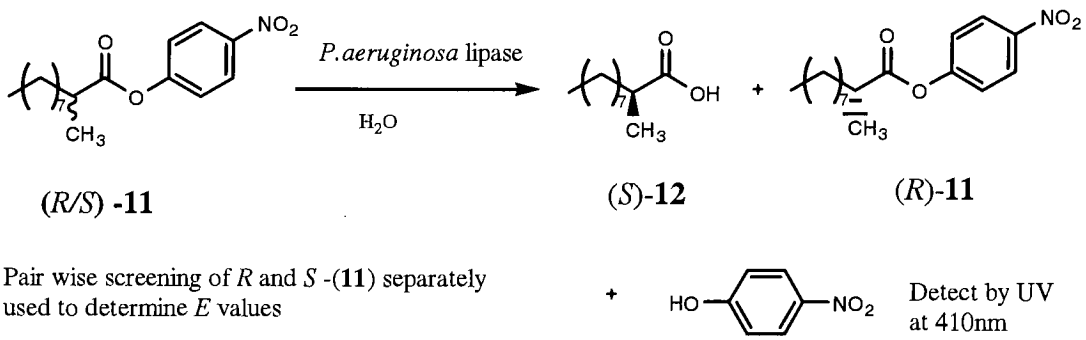
The *p*-NB esterase mutants were screened, in 96 well plates, against a *p*-nitrophenyl ester of Lorcabef. The *p*-nitrophenyl ester is more conveniently screened (at 405nm) than the target *p*-nitrobenzyl ester, although the former is chemically more active. This procedure does violate the first law of directed evolution, coined by Arnold herself, namely - 'You get what you screen for'; although the compromised accuracy is more than compensated for by the increased throughput of the screen.

The overall result of four rounds of sequential mutagenesis and one recombination step was a 50-60 fold increase in activity – this increase also accounts for the increased expression level, which was also achieved. The best mutant esterase performed as well in 30% DMF/H_2O as the wild type enzyme in H_2O. In addition, the effective amino acid substitutions do not directly interact with the substrate, further emphasising the difficulty in mimicking this success by 'rational design'.

Further developments on *p*-NB esterase from *B. subtilis* were made by Giver *et al.*.[59] In this study a 14°C increase in the melting temperature (Tm) was achieved after

four cycles of random mutagenesis and one of DNA shuffling. Screening was carried out for thermostability and activity at 30°C. This screening technique allowed a variant which was both thermostable and, retained its catalytic activity at low temperatures to be identified.

In the area of enantioselectivity, several excellent examples of evolved lipases have been published. Reetz and co workers have published several reports[60-65] on the evolution of a lipase from *Pseudomonas aerginosa* (PAL). The wild type form of PAL displays poor enantioselectivity (E =1, (S)-**12** with 2% e.e.) for the resolution of 2-methyldecanoic acid *p*-nitrophenyl ester **11** - **Scheme 12**.



Scheme 12: *Resolution of 2-methyldecanoic acid p-nitrophenyl ester*

A simplified version of the overall directed evolutionary process[60] is shown in **Figure 6** and some of the improvements are discussed. Initially, four successive rounds of random mutagenesis by error-prone PCR gave a mutant lipase with an increased selectivity factor, E=11.3 (mutant A). [65] Further, more comprehensive directed evolution studies using site-specific saturation mutagenesis (applied at 'hot-spots') increased the E value to 20 (mutant B). A further round of error prone PCR gave a final mutant with an E value of 25 (mutant C).[61] Mutant C had five mutations S149G, S155F, V46G, V55G and S146G compared to the wild type. In addition, elucidation of the 3D structure of wild type PAL indicated that mutant C had much increased flexibility, in distinct loops

of the enzyme, due to the introduction of four glycine residues.[61] Subsequent rounds of error-prone PCR did not give any significant improvements.

In further studies, the application of the recombinant methods, DNA shuffling and combinatorial multiple-cassette mutagenesis (CMCM),[60] gave a final mutant enzyme with an *E* value >51 (mutant J). This mutant consisted of six amino acid substitutions (D20N, S53P, S155M, L162G, T180I and T234S) compared to the wild type.

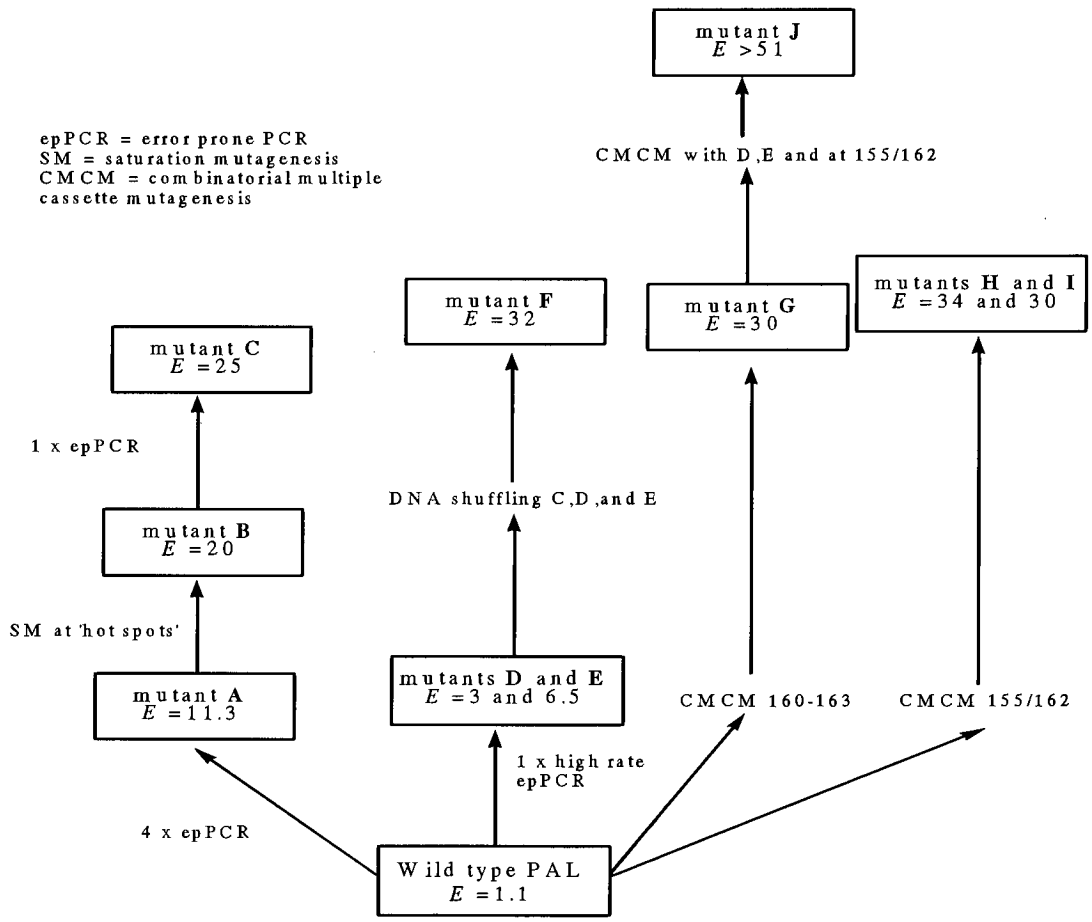
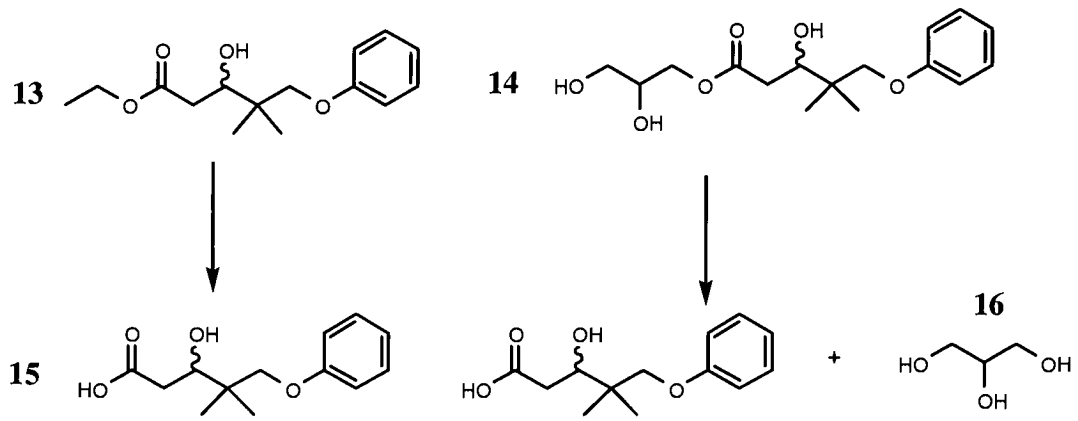


Figure 6: *Summary of directed evolution of PAL by Reetz and co-workers*

In an extension of their work on PAL, Reetz *et al.*,[64] inverted the direction of enantioselectivity from (*S*)-**11** towards (*R*)-**11** - **Scheme 12**. Using error prone PCR, at a high mutation rate, and DNA shuffling a mutant PAL with an enantioselectivity factor *E*=30 towards (*R*)-**11** was created. The new mutant had 11 amino acid substitutions

suggesting that *R*-selectivity required greater structural change than *S*-selectivity. These mutations were also at different 'hot-spots' than had been identified for the *S*-selective variants. This extensive level of mutation would be difficult to predict by molecular modelling.

Continuing on the same theme, Bornscheuer *et al.* [66] foccussed on improving the enantioselectivity of an esterase from *Pseudomonas fluorescens* (PFE) towards the resolution of the 3-hydroxy esters **13** and **14 - Scheme 13.**

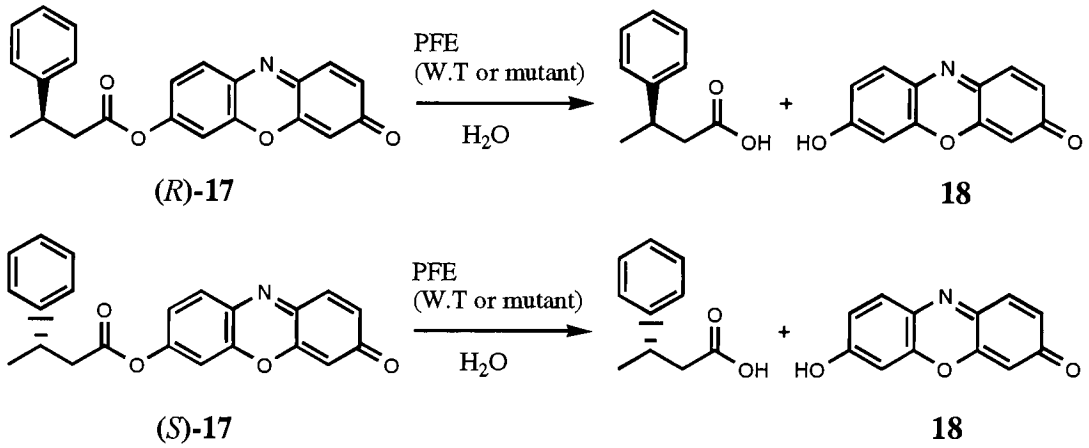


Scheme 13: *Resolution of sterically hindered 3-hydroxy esters* **13** *and* **14**.

Mutations of PFE were acheived using the mutator strain *E. coli* XL1 epicurian red and the enzyme libraries created were screened using pH indicators on minimal media agar plates. Hydrolysis of **13** releases the acid **15**, which lowers the pH of the surrounding media, thus producing a red colour with the pH indicator. A second selection criterion was introduced using ester **14**, which is hydrolysed to release glycerol **16**. The released glycerol facilitates growth on the minimal media. By combining both selection criteria, only larger colonies surrounded by red halos, were picked for further examination. Overall, one double mutant was identified which gave a 25% e.e. for the remaining ester **13** (*E*~5).

In a second study, Henke and Bornscheuer[67] mutated PFE, this time by error prone PCR or mutator *E. coli.*, to increase the enantioselectivity from *E* =3.5 to 6.6. Again the useful mutations were found to be located on the surface of the enzyme, far

away from the active site. On this occasion, screening was performed using resorufin esters of (*R*) and (*S*)-3-phenylbutyric acid **17** with detection by fluorescence - **Scheme 14**. In common with reports by Reetz,[60-65] the (*R*) and (*S*) esters must be screened pairwise in separate wells because it was impossible to determine which enantiomer liberated the resorufin **18**. The E_{app} values were compared with the E_{true} value determined for the hydrolysis of (*R/S*)-3-phenylbutyric acid methyl ester and found to differ only slighty (*E* +/-0.3).



Scheme 14: *Screening of P. fluorescens esterase mutants using resorufin esters*

Other recent publications documenting the evolution of an enantioselective aldolase[68], hydantoinase[69] and a lipase[70] (with increased phospholipase activity) have been made. In addition, several informative reviews, which cover other properties of enzymes which can be manipulated, have also been published.[15, 39, 49, 57, 71, 72]

1.3 Screening novel enzymes

Screening novel enzymes can have two different meanings:

1. Searching for new enzymes in commercially available enzyme libraries, collections of micro-organisms/culture sources or from clone banks.[3]

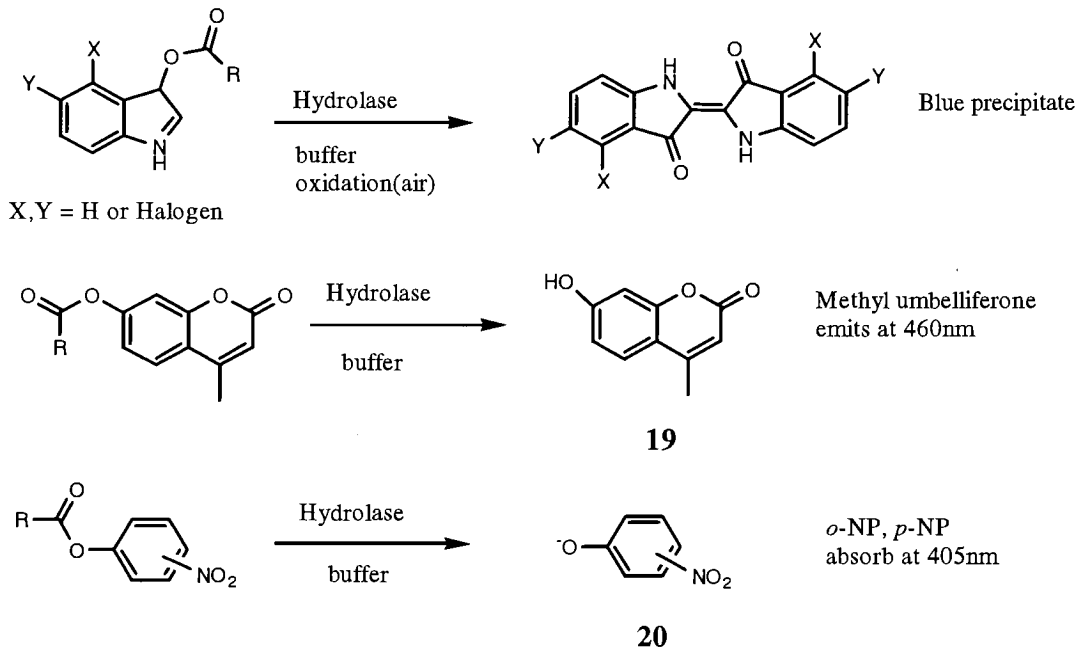
2. Testing a library of mutant enzyme variants generated from directed evolution/site-directed mutagenesis.

In both cases, the target is the same, namely to quickly and accurately discover a more suitable enzyme for the synthetic challenge. In addition, another decision must be made, to screen by detection or to select for the desired properties. Genetic selection is a powerful technique for isolating new mutants as only those that pass a specific test are considered. This approach includes the use of the substrate of interest as the sole carbon source, phage display and suicide inhibitors. These selection techniques have been recently reviewed[20, 73] and phage libraries using a mechanism-based inhibitor have been used for the *in vitro* selection of Lipolase® variants.[74]

When enantioselectivity is the property being measured, selection is not generally possible and individual bacteria or microorganisms must be separated and assayed. Usually a hierarchical (multi-tiered) screening approach[3] is applied but newer automated high-throughput and ultra-high throughput screens[21] allow quantitative screening of every enzyme. Some of the techniques commonly applied are discussed in **Section 1.3.1**.

1.3.1 Agar plate based screening

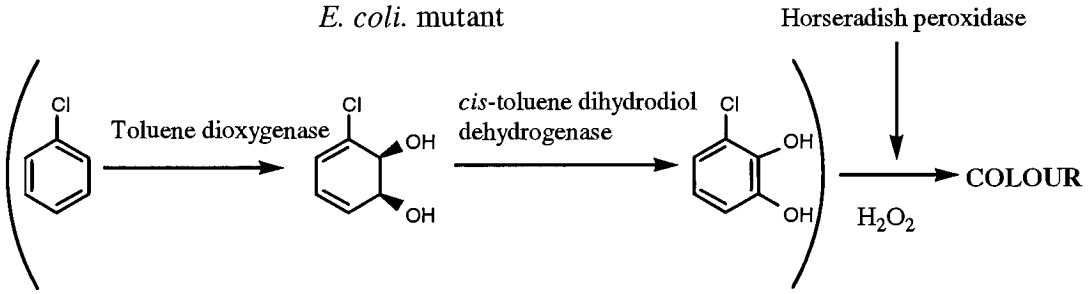
This qualitative technique requires a good system for visualising positives. Several methods have been employed including: the use of substrates which upon hydrolysis release precipitable coloured product[75], detection of the released acid by pH indicator colour changes[66] and the use of tributyrin emulsions.[75, 76] Some examples of useful substrates are given - **Scheme 15.** It must be noted that the *p*-nitro phenolate anion **20** is water soluble and less sensitive for on-plate assays than the fluorescent methyl umbelliferone **19**.



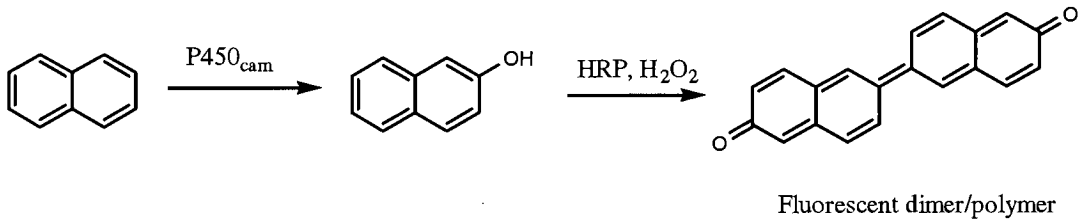
Scheme 15: *Useful compounds for on-plate detection*

Other methods of on-plate assays involve detection of the hydrolysis products by the use of simple staining systems. Ninhydrin[77] and 4-*p*-nitro benzyl pyridine[78] have both been successfully used to detect the products generated by lactamases and epoxide hydrolases, respectively.

Perhaps the most elegant examples of on-plate assay systems have been developed by Arnold and co-workers.[79, 80] By combining the oxygenase-catalysed hydroxylation of aromatic substrates, with the ability of horseradish peroxidase (HRP) to oxidatively couple phenols and catechols, an efficient *in vivo* or *in vitro* screen for $p450_{cam}$ and toluene-dioxygenase variants was developed. For rapid screening *in vitro*, the HRP may be added to the medium containing the dioxygenase-catalysed reaction and assayed using a 96-well plate reader. Alternatively, the HRP can be co-expressed with the oxygenase to create an *in vivo* pathway for the formation of the coloured/fluorescent compounds. The libraries can then be screened on the agar plate using digital imaging or sorted using a microfabricated fluorescence activated cell sorter (FACS).[81] Overviews of the *in vitro* and *in vivo* processes are shown in **Scheme 16** and **Scheme 17**, respectively.



Scheme 16: *In vitro di-oxygenase assay.*



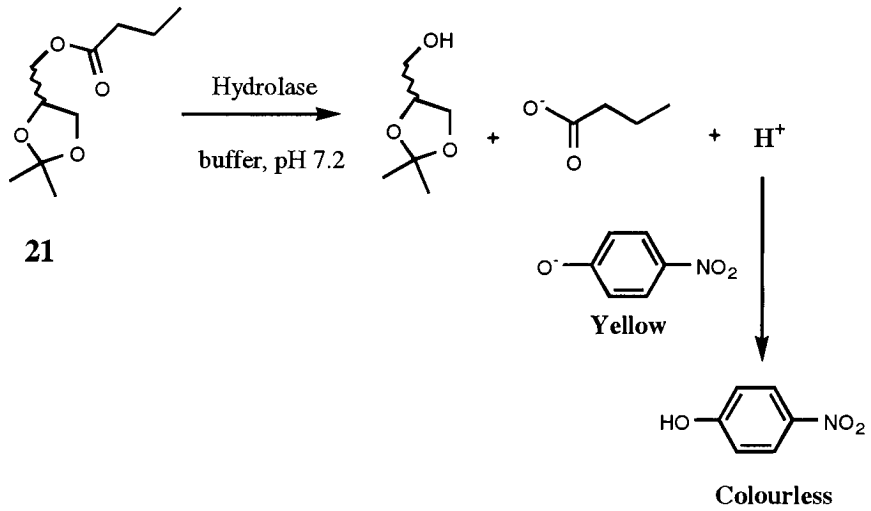
Scheme 17: *In vivo P450cam screen suitable for on-plate assays*

In addition, the screen is sensitive to the regiospecificity of hydroxylation with different hydroxylated products generating different colours. This can be especially useful to quickly identify cells displaying altered product profiles *e.g.* wild type $P450_{cam}$ generates only blue fluorescence (naphthalene converted to 1- or 2-naphthol) but one mutant $P450_{cam}$ clone exhibited pink fluorescence (naphthalene converted to 1,3-dihydroxynapthalene).

1.3.2 pH indicator methods

The use of pH indicators or pH changes (pH-stat method) to monitor the progress of enzyme reactions is not new; researchers have been using this technique since the 1940's. However, by careful choice of the reaction buffer, the colour change of the indicator can be directly proportional to the number of protons released. This technique was first employed by Kazlauskas *et al.*[82] for the quantitative measurement of the hydrolysis of solketal butyrate **21** - **Scheme 18**. 72 commercially available hydrolases

were screened using *p*-nitrophenol indicator, in conjunction with a UV spectrometer, and estimated enantioselectivity ratios calculated. Using this pH indicator technique horse liver esterase was identified as the 'best' hydrolase with an estimated E =12.2 and a true E of 14.8.



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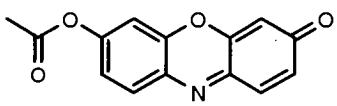
Scheme 18: *Hydrolysis of solketal butyrate and detection using p-nitrophenol indicator*

This pH-indicator method was improved by Kazlauskas *et al.*[51] by the introduction of a reference. Resorufin ester **22** was included as a competitive substrate. The improved version was implemented in the substrate mapping of 4 new hydrolases.[83]



22

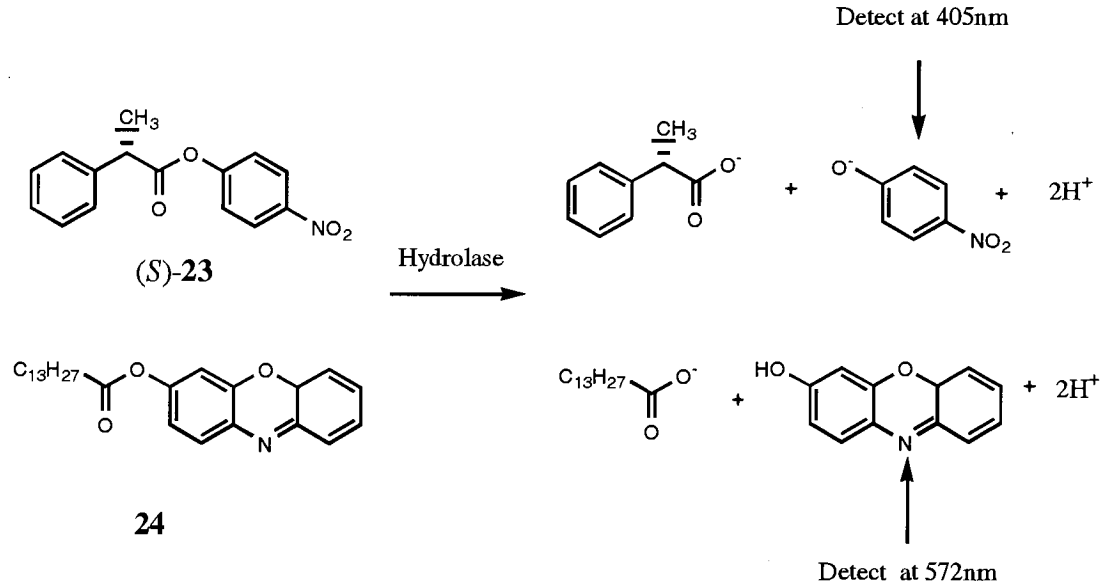
A similar, although non-quantitative approach was applied by Demirjian.[84] This screen required careful matching of the indicator and the pH of the buffer. This method would be useful as the first level in a hierarchical screening process. Bornscheuer *et*

al.[85] employed bromothymol blue as the first level of a hydrolase screen, with any positive 'hits' later confirmed by quantitative GC analyses.

1.3.3 Chromogenic substrates

The most convenient reactions to test by high-throughput methodologies are those involving chromogenic or fluorogenic (discussed in **Section 1.3.4**) substrates. There are countless reports on the application of chromogenic substrates to screening enzymatic reactions, in which the released alcohol or carboxylic acid is UV active. Many of these have been covered in an excellent review[75] by Beisson *et. al.*.

One of the most widely used chromogenic substrates are esters of *p*-nitrophenol and 2,4-dinitrophenol. The former was used as the substrate in the directed evolution of a lipase (**Section 1.2.3**) and forms the basis of the Quick *E* method reported by Kazlauskas *et. al.*[86] Quick *E* measures the rate of hydrolysis of an enantiomerically pure *p*-nitrophenol ester (*S*)-**23**, against the reference compound, resorufin tetradecanoate **24** - **Scheme 19**. The ratio of these rates gives the selectivity of the hydrolase towards (*S*)-**23** over **24**. In tandem with this, the selectivity of the hydrolase for (*R*)-**23** is also measured against **24**. The ratio of the selectivities gives the enantiomeric ratio, *E*.



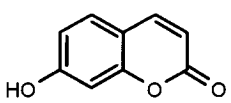
Scheme 19: *Quick E method*

Inspite of these succesful reports, several arguments against the use of *p*-nitrophenol esters have been presented[75]:

1. They are not specific lipase substrates and can be hydrolysed by non-specific esterases, proteases and moreover, the non-catalytic *C*-terminal domain (336-449) of porcine pancreatic lipase (PPL) can hydrolyse *para*-nitrophenol acetate at the same rate as purified PPL.[87]
2. *p*-nitrophenol acyl esters are tertiary esters whereas most lipases act on primary ester bonds.
3. The carbonyl function of these compounds is activated and therefore likely to undergo non-enzymatic hydrolysis in alkaline or acidic media.

1.3.4 Fluorogenic substrates

An attractive property of fluorogenic substrates is the high sensitivity with which the hydrolysis products can be detected. This makes them particularly attractive when either: substrate concentration is very dilute, a small amount of biocatalyst is available or low substrate turnover is to be measured. The majority of assays have employed simple esters of either umbelliferone[88] **25** or resorufin[67] **26** as the substrate. However, much like simple *p*-nitrophenol esters these esters are highly chemically activated and as such may lead to erroneous results.



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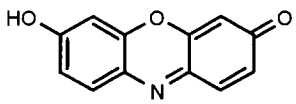

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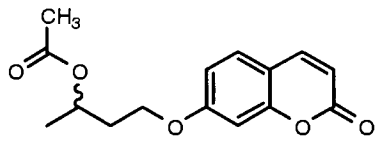
Klein and Reymond have developed[89] fluorescent lipase/esterase substrates **27/28** which circumvent this high chemical reactivity but retain the high sensitivity of **25**.



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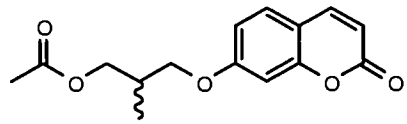
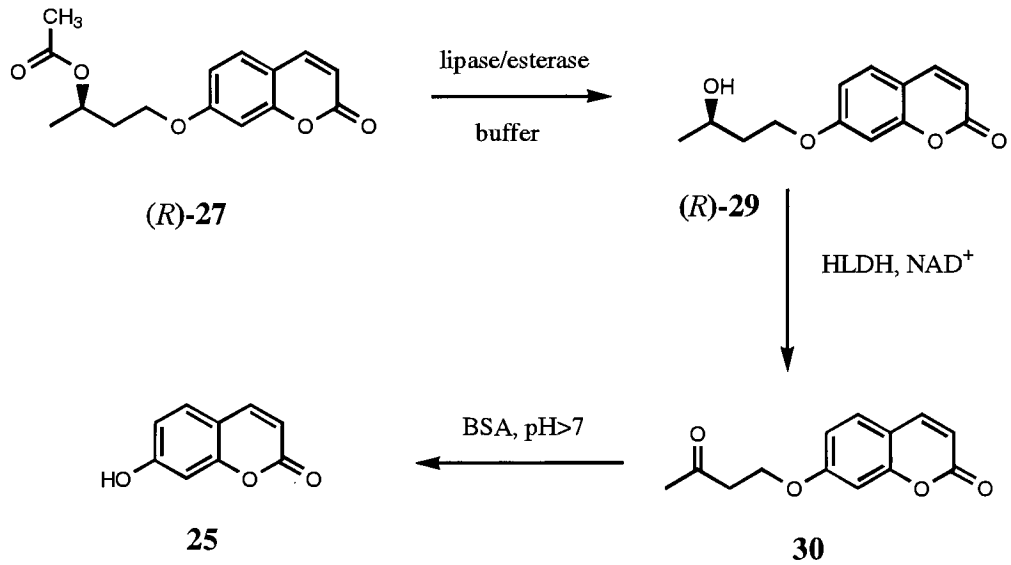


28

Considering the example of ester (*R*)-**27**, the alcohol **29** (released by enzymatic hydrolysis of this acetate) is oxidized by horse –liver alcohol dehydrogenase (HLDH) to an unstable ketone **30**. This ketone then undergoes β-elimination, catalysed by bovine serum albumin (BSA), to release umbelliferone **25**. The overall process (for (*R*)-**27** only) is shown - **Scheme 22**. A similar mechanism occurs for **28**.



(*R*)-**27**

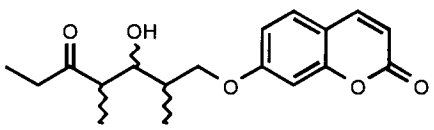
(*R*)-**29**

25

30

Scheme 22: *Release of umbelliferone* **25** *from (R)*-**27**

By measuring the release of **25**, from (*S*)-**27** and (*R*)-**27** separately, an estimated enantiomeric ratio *E* can be predicted. The authors predicted *E* values for 30 enzymes and found them to lie within +/- 20% of the true value. By modification of the fluorogenic substrates and the assay conditions, Reymond *et. al.* have developed similar substrates **31** and **32** for stereoselective aldolases [90] and other hydrolytic enzymes (acylases *etc.*),[91] respectively.



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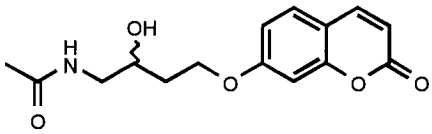


32

Continuing with fluorescent assays, Reymond and co-workers developed[92] an anti-body sensor assay for monitoring catalysis in real time. This system is based on the cat-ELISA technique developed by Green[93] and Hilvert,[94] although these systems only furnished a single-point measurement of product formation. The principle of Reymond's assay is displayed - **Figure 7**.

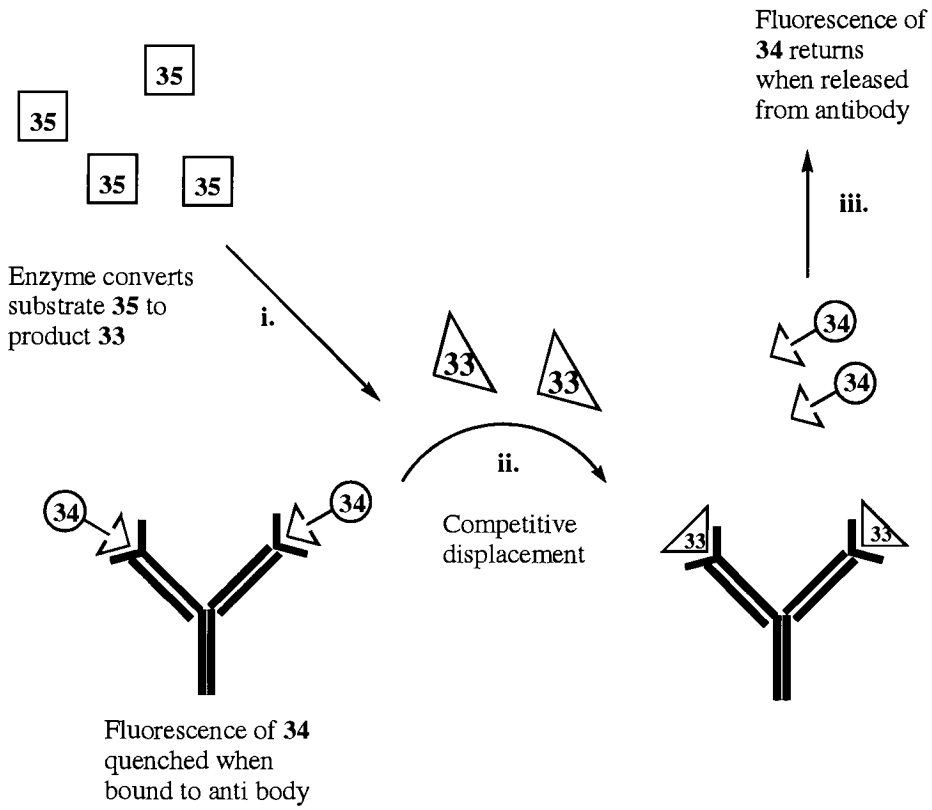
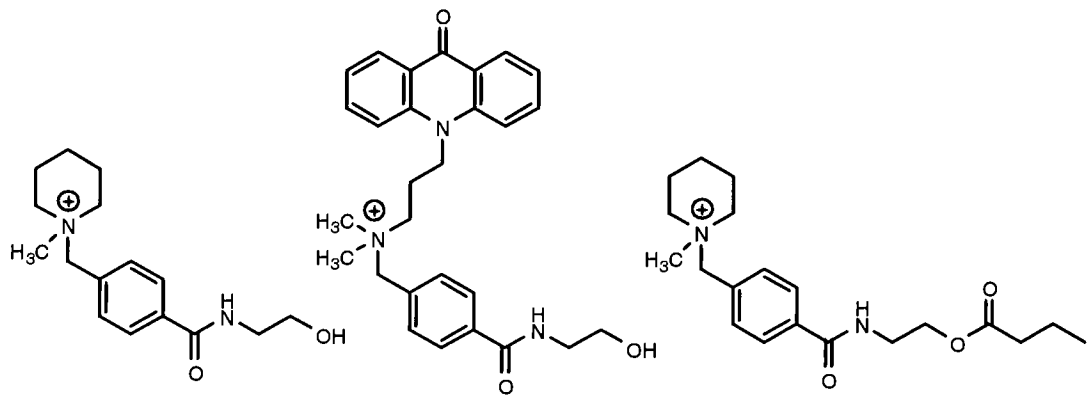


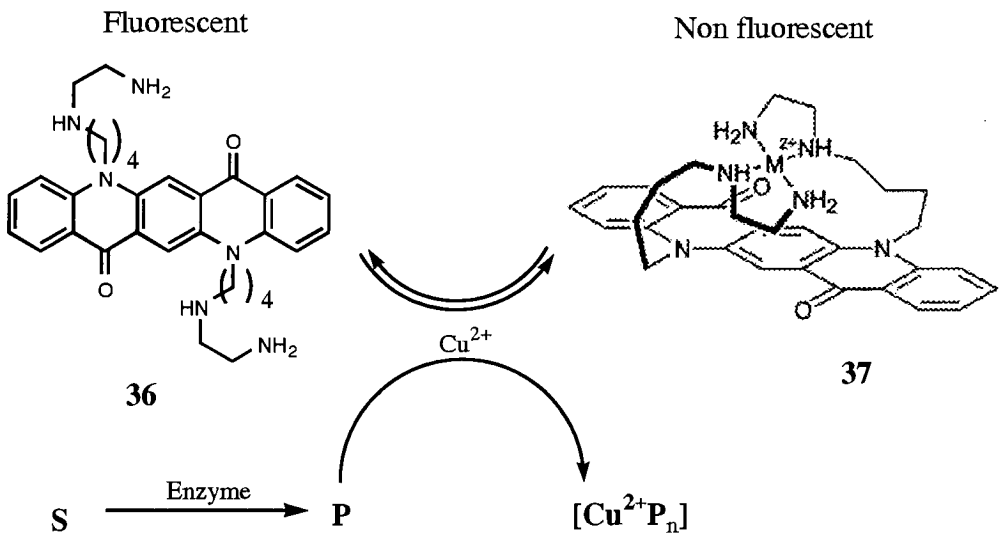
Figure 7: *i. Enzymatic reaction, ii. displacement of* **34** *by* **33**, *iii. detection of released* **34**.



33 **34** **35**

Antibodies that can bind hapten **33** with high selectivity over the derivative **35** (which is a substrate for the enzyme to be measured) were developed. A selective fluorescent sensor for hapten **33** was prepared by combining anti-**33** antibodies with **34**, an analogue of **33** containing the fluorescent tag acridone. The fluorescence of **34** is quenched while bound to the antibody, although fluorescence returns when **34** is released. Hog liver esterase hydrolysis of **35** released **33** (**step i, Figure 7**) which subsequently displaced **34** (**step ii, Figure 7**), and the fluorescence generated was measured (**step iii, Figure 7**). The rates of product release were calculated and the apparent reaction rates were proportional to enzyme concentration at low catalyst concentrations. The system was also applied to the measurement of β–galactosidase activity using modified substrates.

Reymond and Klein[95] have developed another fluorescent sensor system, this time based on measuring the change in concentration of free metal ions using the orange fluorescent metal sensor **36**. **36** responds to the prescence of Cu^{2+} ions, forming the macrocyclic chelate **37** – **Scheme 23**. This is accompanied by an almost quantatative quenching of fluorescence. Any enzymatic reaction, which produces products that can act as a metal chelator, such as simple bi-dentate ligands *e.g.* amino acids, can compete with **36** for the free metal ions. This system has been used to monitor the release of free amino acids using acylases and aminopeptidases.



Scheme 23: *Enzymatic assay using pM*

The use of fluorescence resonance energy transfer (FRET) substrates have been used to study a range of catalysts including ribozymes[96], proteases using a combinatorial library[97] and phospholipases[98]. FRET substrates have also been used in conjunction with fluorescence activated cell sorting to screen a library of mutant proteases.[20]

1.3.5 Instrumental methods

The demand for screening large compound collections against an ever-expanding number of therapeutic targets has stimulated technological development in the area of assay automation, miniaturization and detection methodologies.[21] Assay throughput was initially addressed by the use of 96-well plates and the development of automated sample preparation including robotic colony pickers and liquid-dispensing technologies. Throughput was further increased with the introduction of 384-well plates and the trend is continuing towards higher density, smaller volume plate formats. In addition, the development of microfabricated devices designed to perform continuous flow assays, offer the potential to achieve the following advances; order-of –magnitude reductions in reagent consumption, parallel sample processing for higher throughput, implementation

of unique assay read-outs and improved data quality.[21] These techniques have allowed ultra-high throughput rates in excess of 100,000 samples per day and will soon become more widespread in the screening of mutant enzyme libraries. At present however, the screening of enzyme libraries, for more enantioselective mutants, generally relies on the modification of more-traditional techniques (HPLC, GC and MS) into higher throughput formats.

1.3.5.1 Chromatographic techniques

Reetz et al[99] have described a GC system capable of high-throughput screening of mutant lipases for improved enantioselectivity. This system uses two gas chromatographs (both fitted with chiral columns) controlled by one sample manager and PC - **Figure 8**. The GC system was capable of approximately 700 exact *E* and e.e. determinations per day and the analytical data can be manipulated using commercially available software. Manipulation of the injection timing can further increase throughput by interlocking the chromatograms and utilising all available instrument time but this technique requires a degree of trial and error.

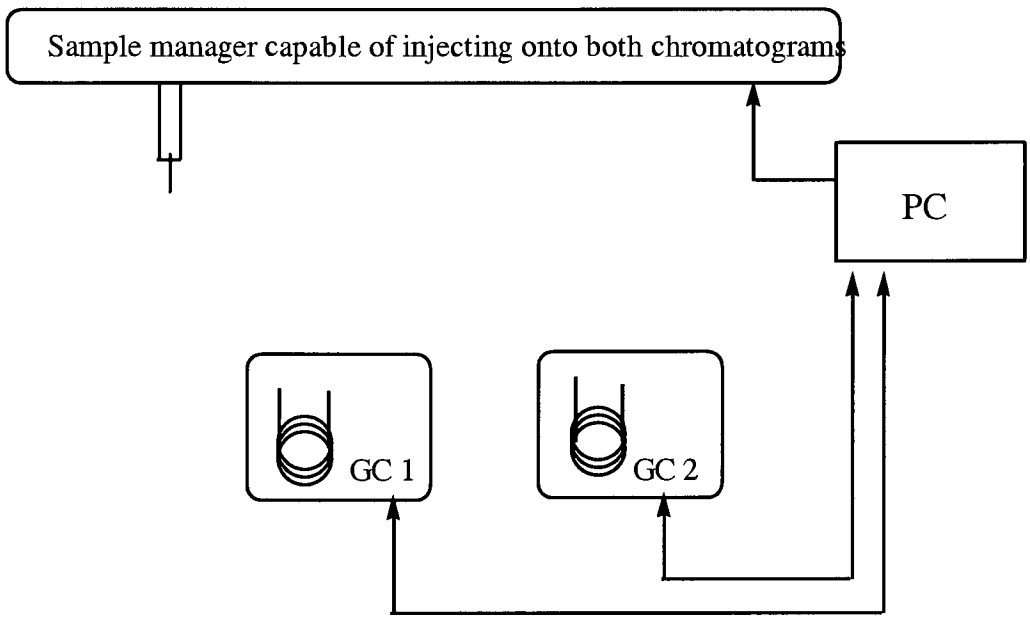
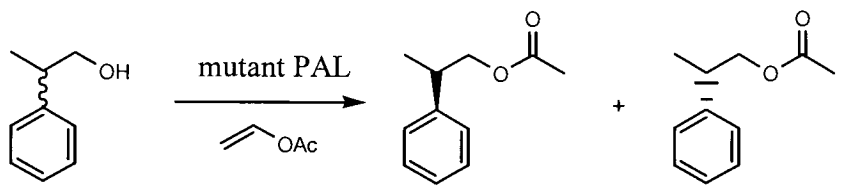


Figure 8: *Optimized twin GC unit for high throughput GC analyses.*

Using this system (**Figure 8**), Reetz et al[99] studied the lipase-catalysed stereoselective esterification of (+/-) 2-phenyl-1-propanol **38** by a library of mutant *Pseudomonas aeriginosa* lipases (see **Section 1.2.3**) to give the corresponding esters **39** - **Scheme 24**.



(*R/S*)-**38** (*R*)-**39** (*S*)-**39**

Scheme 24: *Acylation of 2-phenyl-1-propanol catalysed by mutant P. aeriginosa lipases.*

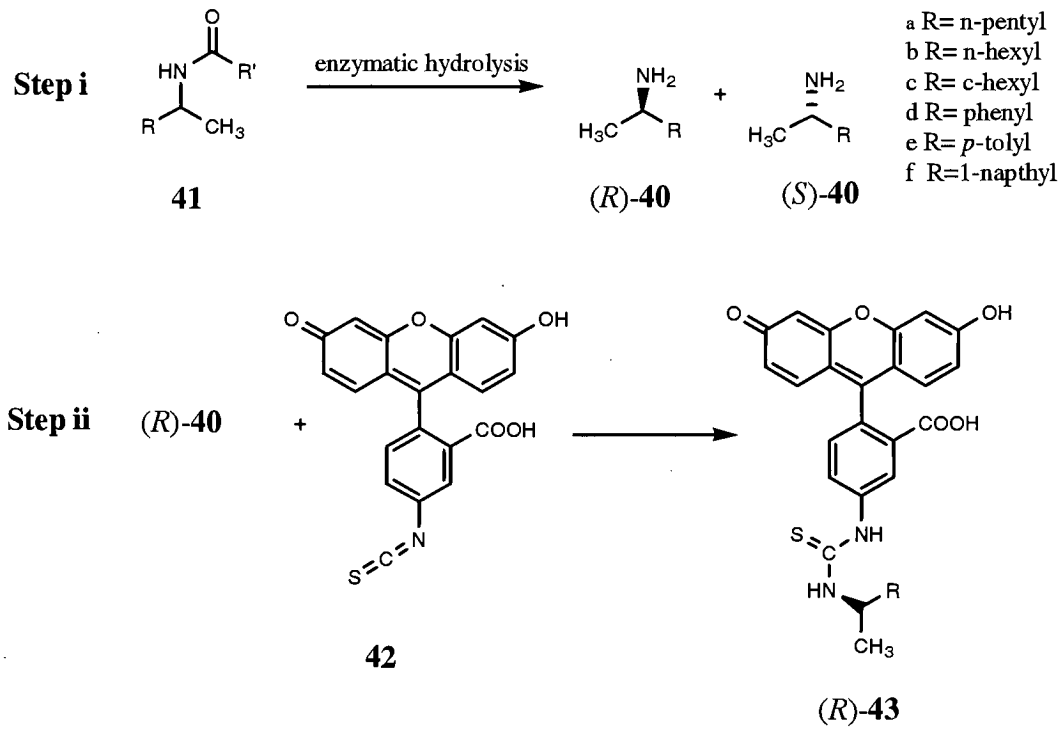
Reetz[19] also suggested the use of chiral thin layer chromatography to measure enantioselectivity. Rapid screening using TLC, would require computer image processing of the chiral TLC plates to determine the relative sizes of the separated enantiomers of product and starting material. As yet, no reports of enzyme screening using TLC have been published.

1.3.5.2 Electrophoretic techniques

The introduction of commercially available capillary array electrophoresis (CAE) instruments and capillary electrophoresis (CE) on microchips has allowed CE to be applied to high throughput screening. The introduction of chiral modifiers to the running buffer allows the determination of enantiomeric purity and makes either technique suitable for screening mutant enzymes.

Reetz *et. al.*[100] utilized an automated 96 array CAE system, with laser-induced fluorescence (LIF) detection, to measure the enantiomeric ratio of known mixtures of chiral amines. A range of chiral amines (*R*)-**40a-f** and (*S*)-**40a-f** were investigated. In principle, these amines could be generated by enzymatic hydrolysis of the appropriate acetamide **41** - step **i**, **Scheme 25**. To facilitate fluorescent detection, the free amines were dervatised using fluorescene isothiocyanate **42** to give the fluorescence active

derivatives (*R*) and (*S*)- **43** (step **ii, Scheme 25**). The overall procedure is shown in **Scheme 25,** with only (*R*)- **43** shown for clarity. By careful optimisation of the running buffer, (*R*) and (*S*)-**43** can be easily separated. A typical run-time of 19 mins (for (*R*)+(*S*)-**43c**) was achieved allowing more than 7000 e.e. determinations per day. In a similar area, recent reports[101, 102] on the separation of enantiomers of amino acids by CAE on microchips have shown this technique to be applicable to high-throughput enantioselectivity determinations.



Scheme 25: *i Enzymatic resolution of* **41***; ii. pre-capillary derivatisation of* (*R*)-**40**

1.3.5.3 Mass-Spectrometric assays.

Reetz and co-workers[103] have developed a high-throughput mass-spectrometric screening technique for enantioselective catalysis. The underlying principle is based on the use of isotopically labeled substrates in the form of *pseudo*-enatiomers or *pseudo*-prochiral compounds. The relative amounts of reactants and/or products were detected

by electrospray mass spectrometry (ESi-MS). This technique is applicable to several different asymmetric transformations – **Figure 9**.

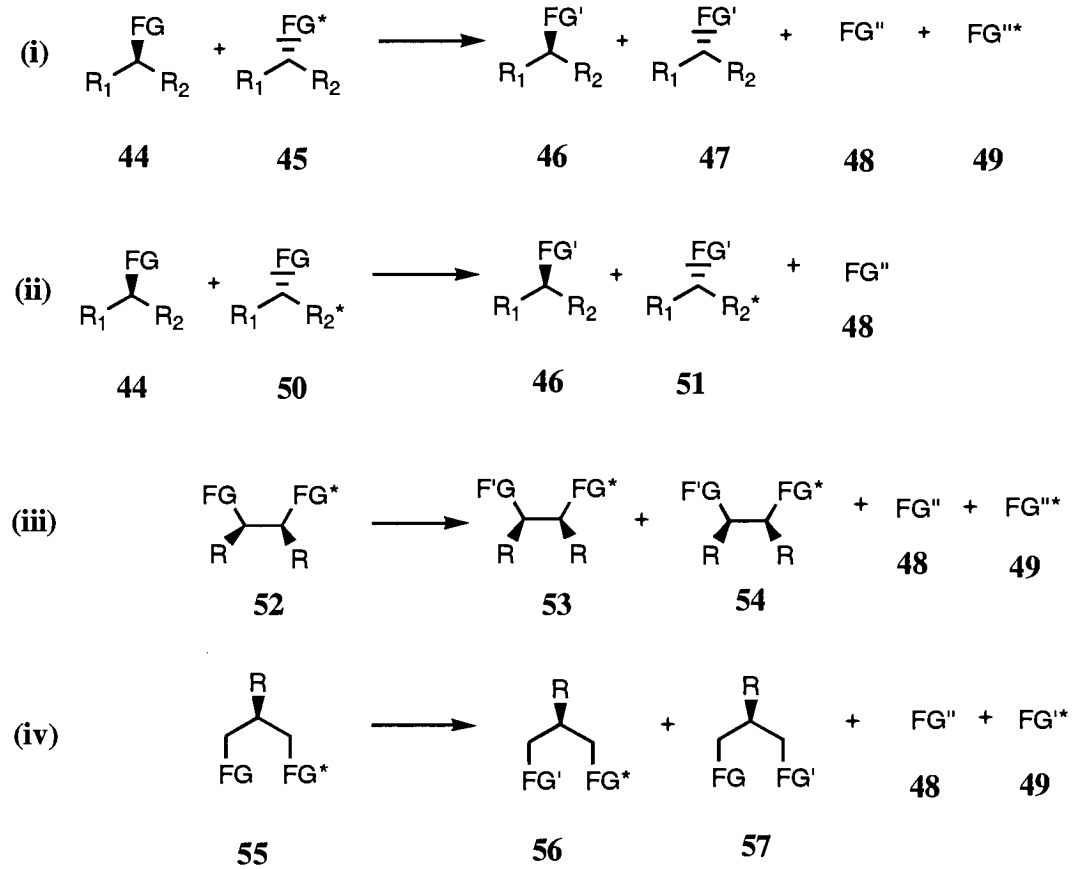


Figure 9: *Application of mass-spectrometry to measurement of enantioselective catalysis*

Figure 9 (i) represents a typical enzymatic kinetic resolution: compounds **44** and **45**, which differ in absolute configuration and in isotopic labelling of the functional group (FG*), are synthesized independently and mixed to give a racemic mixture. After resolution, true enantiomers **46** and **47** are formed as well as the nonlabeled and labelled achiral products **48** and **49**. The ratio of the total intensities **44/45** and **48/49** allows determination of the enantioselectivity.

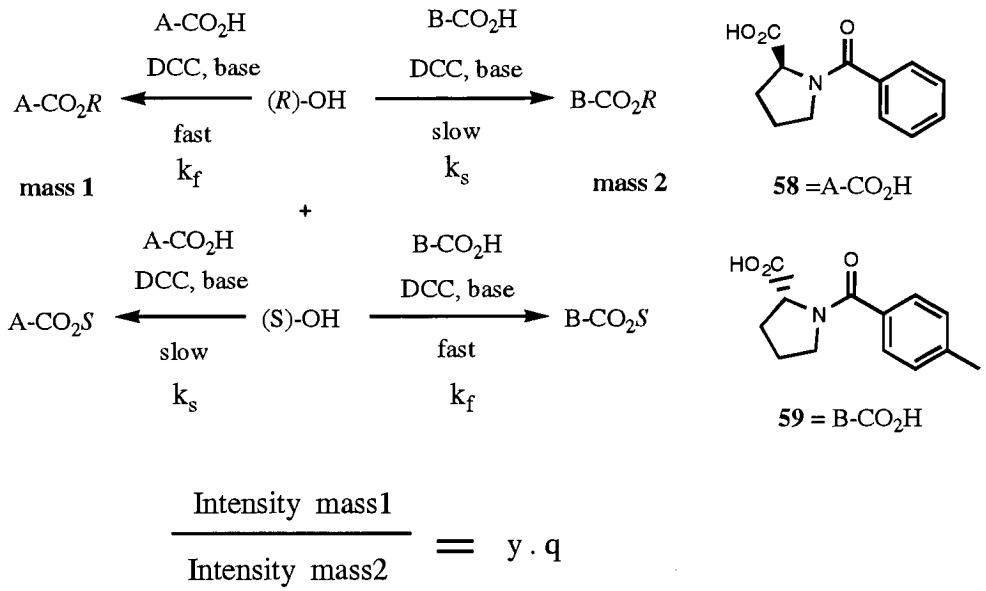
Figure 9 (ii) represents a typical kinetic resolution similar to **(i)** except in this case labelling occurs at residue R_2. In this case, using the m/z intensities of the *pseudo-*

enantiomers **44/50** and **46/51** allows the conversion, enantioselectivity and the selectivity factor to be determined.

Figure 9 (iii) and **(iv)** represent the resolution of *pseudo-meso* and *pseudo*-prochiral substrates, respectively. In both cases only a single labelled compound is required (*e.g.* **52**) since the stereodifferentiating reaction delivers a detectable mixture of two *pseudo*-enatiomers **53** and **54**.

Reetz and co-workers[103] applied this system to study three different lipase-catalysed reactions; hydrolytic kinetic resolution, stereoselective esterification and the enantioselective hydrolysis of a *meso* compounds. In all cases, the results achieved by ESi-MS were comparable with those determined by GC and throughput was approximately 1000 samples per day.

Finn and co-workers[104] applied a different approach to the determination of e.e. values of alcohols. It is based on dervatization of the alcohol using chiral mass-tagged acylating reagents, and requires a measurable degree of kinetic resolution during the derivatization step. This idea was first proposed by Horeau and co-workers in several reports.[105-108] In general, the reactions of enantiomers with any pair of chiral reagents will proceed with non-equal rates ($k_f > k_s$; f=fast and s=slow) - **Scheme 26. Scheme 26** displays the reaction of different enantiomers of an alcohol with two chiral mass tagged acids, A-CO_2H and B-CO_2H. In this report, Finn *et al.* [104] used the two mass-tagged proline derivatives **58** and **59**. The relative amounts of the product esters, measured by mass spectrometry, can be used to determine the enantiomeric composition of the starting substrate according to **Equation 1** and two separate calibration measurements. Calibrations measurements must be performed on a racemic sample and a sample of known enantiomeric excess. Calibration measurements allow **q**, a correction factor for differences in ionization efficiencies between compounds differing by a methyl group, and **s**, the ratio of the rate constants, to be calculated. The enantiomeric excess can then be calculated using **Equations 1** and **2**



$$\frac{\text{Intensity mass1}}{\text{Intensity mass2}} = y \cdot q$$

Scheme 26: *Mass spectrometric measurement of e.e. using kinetic resolution*

$$\%\ \text{e.e.} = \left[\frac{(y-1)\,(s+1)}{(y+1)(s-1)} \right] \text{X } 100$$

$$s = \frac{k_f}{k_s}$$

y = corrected intensity ratio

Equation 1

$$S = \frac{[\ \%\ \text{e.e.}\ (y+1) + 100\ (y-1)\ ]}{[\ \%\ \text{e.e.}\ (y+1) - 100\ (y-1)\ ]}$$

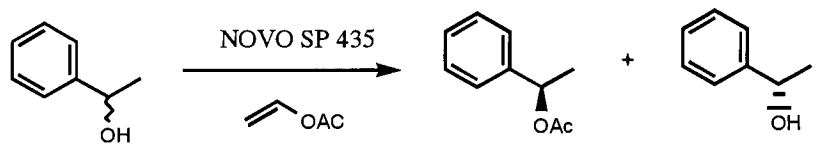
Equation 2

Finn *et. al.*[104] applied this technique to measure the e.e. values of nine secondary alcohols and five primary and secondary amines. All the measured e.e. values fell within +/- 10% of the true value and this method should be amenable to automation and high throughput analysis.

A recent informative review[109] of the applications of mass spectrometry for the screening of new biocatalysts, highlighted advances in instrumentation, analysis strategies and high throughput detection. Three 'real-life' examples were also given.

1.3.5.4 Thermographic assays.

Time-resolved IR thermography was applied by Reetz *et al.*[110] to measure the progress of the *Candida antarctica* lipase (NOVO SP 435) catalysed acylation of 1-phenylethanol **60** - **Scheme 27**. This reaction is known to favour (*R*)-**61** with 99% enantioselectivity.

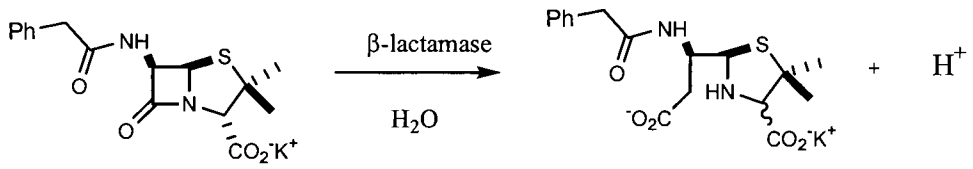


60 (*R*)-**61** (*S*)-**61**

Scheme 27: *Enantioselective acylation of 1-phenylethanol*

Separate reactions of (*R*)-**60**, (*S*)-**60** and (*R/S*)-**60** with vinyl acetate and enzyme were measured in individual wells of a 96-well plate. The results clearly indicated that (*R*)-**60** reacted preferentially with respect to (*S*)-**60,** with those wells containing (*R*)-**60** showing a significantly larger rise in temperature compared with those containing only (*S*)-**60** or (*R/S*)-**60**. This method has yet to be applied in a quantitative fashion, or to reactions in which the enantioselectivity is so marked.

In a similar area, Connolly and Sutherland[111] demonstrated the use of an automated multiplex array of thermistors to measure the temperature change associated with biochemical reactions. In general, thermistors are more sensitive than IR-thermography and can measure reactions in which only a small amount of substrate has been converted to product. This technique was applied to the exothermic β-lactamase catalysed hydrolysis of penicillin G **62** to penicillinoate **63** - **Scheme 28**.

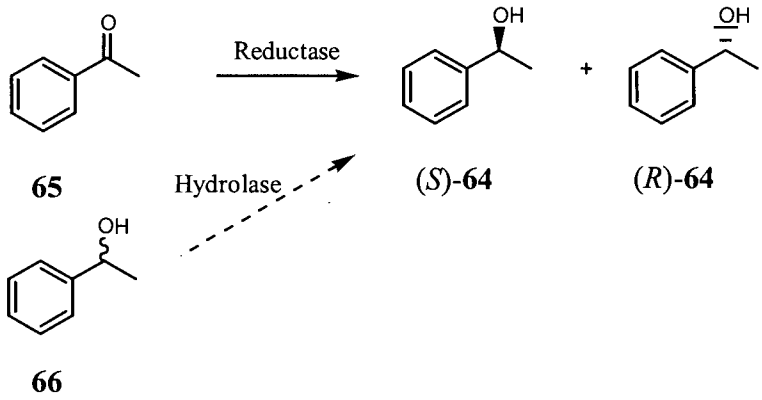


62 **63**

Scheme 28: *β-lactamase hydrolysis of penicillin G* **62**.

1.3.5.5 Other instrumental techniques

High throughput HPLC with circular dichroism (CD) detection has been applied[112] to determine the enantiomeric purity of 1-phenylethanol **64** formed in the reductase catalyzed reduction of acetophenone **65** – **Scheme 29**. (Note **64** could also be formed in a lipase catalysed hydrolysis reaction of the acetate **66**).



Scheme 29: *Enantioselective reduction of acetophenone.*

Studies using enantiomerically pure **64** determined a linear relationship between g and the e.e. value. The rate of sample throughput is dependent on the speed of the separation of the starting material from the product. In the example given - **Scheme 29**, baseline separation was achieved, of **65** from (*R*)/(*S*)-**64**, in less than 1.5 mins allowing 700-900 e.e. determinations per day. CD based assays can also be performed[112] using no chromatographic step, although this relies on the transformation of a prochiral substrate into a UV-active product.

Radioactivity assays[113] and ^{19}F NMR[113] have also been indicated as potential instruments for the rapid determination of enantioselectivity although no high-throughput techniques have yet been developed.

Shair and co-workers[114] have utilised DNA microarray technology to develop a high-throughput fluorescence assay for the determination of the enantiopurity of amino acids - **Figure 10**. This technique consists of five synthetic steps beginning with the formation of *N*-Boc derivatives of the amino acid **67** to be measured. These are then coupled to amino-functionalised glass slides, followed by capping any unreacted amino groups with acetic anhydride. Complete acidic de-protection reveals the free amino group of the amino acid which are then reacted with two *pseudo*-enantiomeric fluorescent probes, **68** and **69**. The fluorescence spectra of the surface bound fluorophores provide the ratio of **68** to **69** and the original optical purity of the amino acid. Using this technique, mixtures of 5 chiral amino acids were measured in steps of 10% e.e. and the values measured were within (+/-) 10% of the actual value. In a high-throughput fashion 15,552 samples were assayed on a single glass slide.

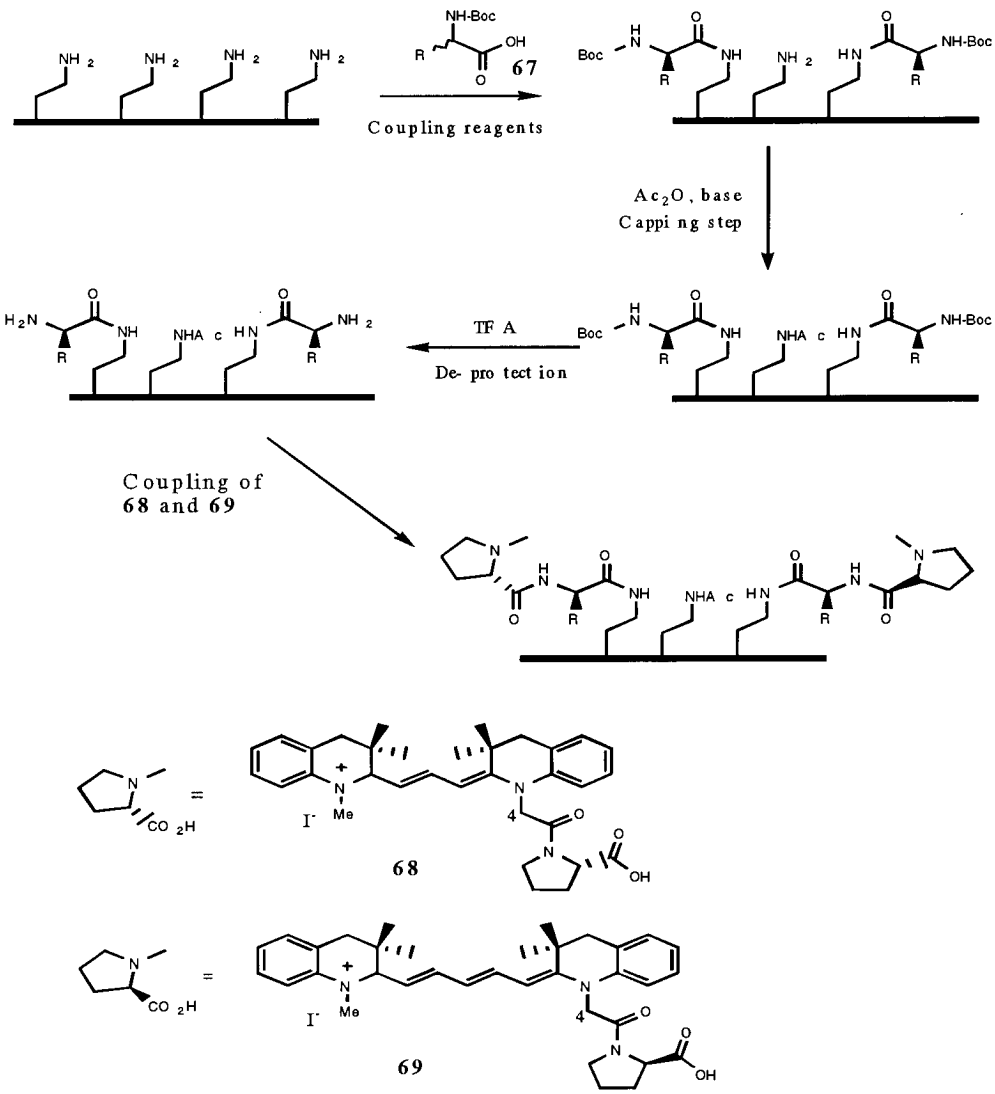
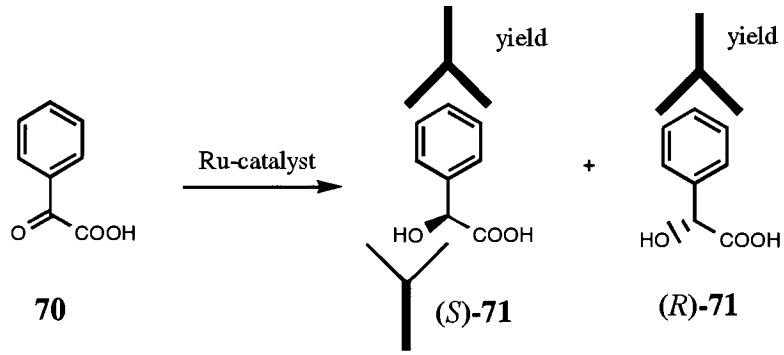


Figure 10: *Microarray determination of enantiomeric purity.*

1.3.6 Biological techniques

As mentioned previously (**Section 1.3.4**), antibodies can be used to monitor enzyme reactions. The development of the competitive cat-ELISA assay by Mioskowki and co-workers[115,116] allowed the testing of the activity and selectivity of a biocatalyst towards substrate. In a similar development by the same group,[117] enzyme immunoassays were applied to measure the e.e. of the product of an enantioselective reaction. The enantioselective Ru-catalysed hydrogenation of benzyl formic acid **70**

gives a mixture of *R* and *S* mandelic acid **71** – **Scheme 30.** By generating two antibodies, one which binds the racemate, and the other, which binds only one enantiomer of **71**, the yield and e.e. value can be determined. This technique can be automated to facilitate 1000 e.e. determinations a day and easily applied to enzymatic reactions.



Scheme 30: *Enzyme immnoassay screening of enantioselective reactions*

The QUEST[118] system (QUerying for EnzymeS using the Three-hybrid system) detects catalysis by coupling substrate turnover to a transcriptional event. This method involves the use of bacterial cells capable of expressing a synthetic transcriptional activator, which can bind the substrate of an enzymatic reaction and a chemical inducer of dimerization (CID) **72**. Dimerization by the CID allows the cells to metabolize arabinose and subsequently acidify the growth media. A pH indicator detects this drop in pH. The addition of substrate competes with the CID for binding to the activator and prevents metabolism of the arabinose. However, if an enzyme, capable of converting the substrate to product, is present then the *in vivo* equilibrium favours CID re-binding of the activator and resumption of arabinose metabolism. The process and structure of the CID used are displayed - **Figure 11**. This technique was used to detect the fungal enzyme scytalone dehydratase in bacteria and was applied to a library of 192 mutant colonies.

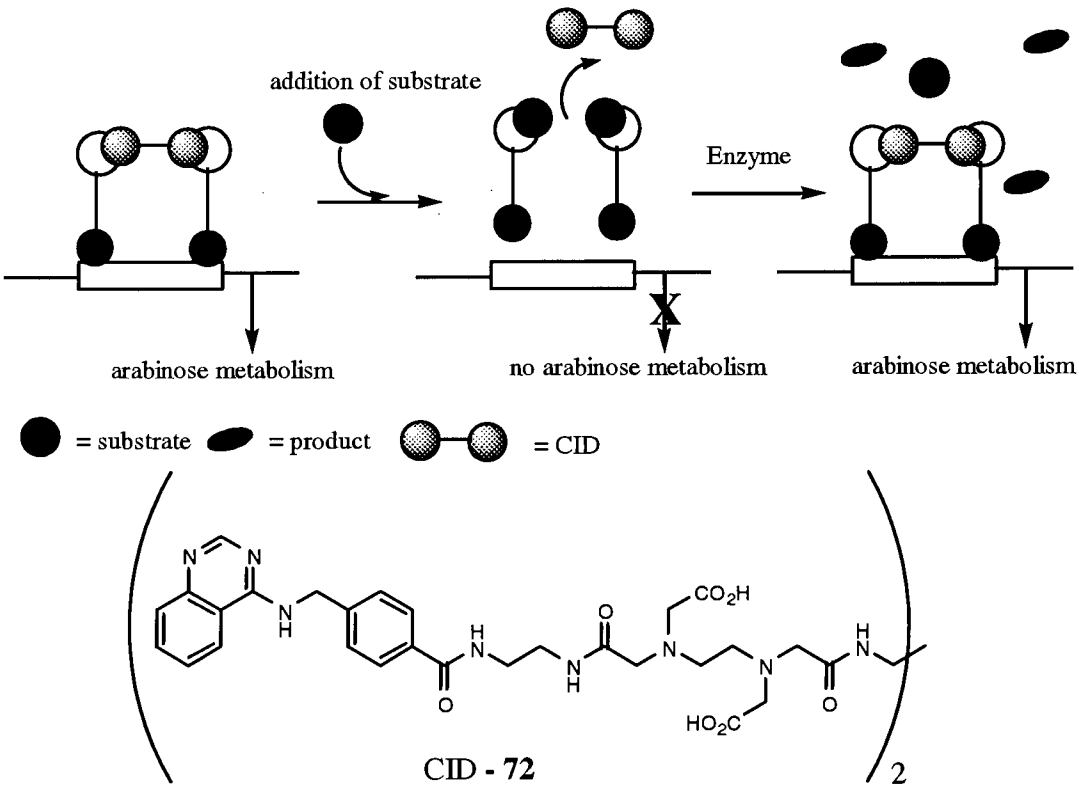
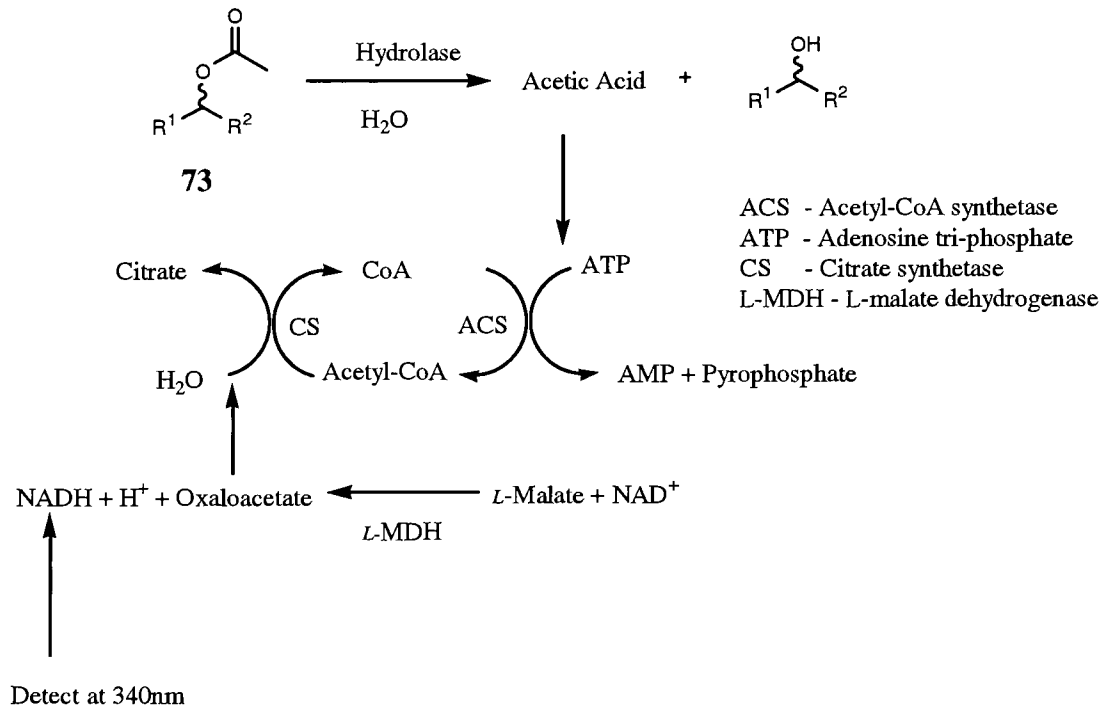


Figure 11: *Schematic of QUEST system and CID-***72.**

Bornscheuer and co-workers[119] have used a cascade of enzymatic reactions to monitor the release of acetic acid, formed in the lipase/esterase catalysed hydrolysis of a methyl ester **73 – Scheme 31**. Initial rates of acetic acid formation can be measured by initial rate of formation of NADH (at 340nm). By measuring the initial rate of NADH formation from (*R*)-**73** and (*S*)-**73** separately, E_{app} can be calculated. The enzyme cascade is supplied from a test kit and about 13 000 mutants can be screened per day. This system was used to screen a variety of lipases/esterases but it use is applicable in any enzymatic reaction which liberates acetic acid. Several other biologically based enzyme screening techniques have been reviewed recently[20] by Olsen *et. al.*.



Scheme 31: *Enzyme-cascade detection of hydrolytic enzymes*

1.3.7 Summary

As can be seen from the previous examples a myriad of solutions can be applied to the screening of biocatalyst libraries. However, the development of further techniques and instrumentation is required because no one technique is likely to be truly universal. One technique which we propose to employ for biocatalyst library screening is Surface Enhanced Resonance Raman Scattering (SERRS).

1.4 Surface Enhanced Resonance Raman Scattering

It is well established[120, 121] that surface enhanced resonance raman scattering (SERRS) is one of the most sensitive and selective techniques for the detection of a wide range of chromophoric analytes. SERRS can provide[120-122] molecularly distinct spectra of each analyte with minimal to no separation. As the name suggests SERRS is a

combination of two spectroscopic techniques; namely surface enhanced Raman and resonance Raman scattering. In the broadest sense it involves excitation of a surface adsorbed analyte, using a laser excitation frequency tuned to the absorption maximum of the analyte, and detection of the resultant Raman scattering.

1.4.1 Raman Scattering

When a photon of light interacts with a molecule it can undergo one of 3 processes almost simultaneously: absorption, absorption followed by emission or scattering. An interaction between the photon and the molecule that results in scattering of the light can occur in two ways: elastic or inelastic. The majority of the photons are scattered elastically and thus emerge at the same frequency as the excitation radiation in a process termed Rayleigh scattering. A small number of photons, about 1 in every million, are inelastically scattered and emerge with a different frequency to the incident photon. This process is known as Raman scattering and it is this radiation which is measured in Raman spectroscopy.

Raman scattering can be described as the re-radiation of scattered light, by dipoles induced in the molecule by the excitation light, which is modulated by the vibrations in the molecule. Therefore, a major advantage of Raman spectroscopy is its ability to provide structural information on a molecule because the frequency of the Raman scattered photons is dictated by transitions between the rotational and/or vibrational energy levels within the molecule. However, the small proportion of photons emitted as Raman scattering means that a large number of analyte molecules are required rendering the technique unsuitable for trace analysis. This inherent lack of sensitivity has been improved significantly in recent years by the introduction of high intensity monochromatic laser sources capable of producing stronger scattering signals.

Raman scattering can be split into Stokes and anti-Stokes scattering. In Stokes scattering, the molecule in the lowest vibrational energy state of the ground electronic state is excited into a short lived virtual state whose energy is equal to the energy of the incident photons ($h\upsilon_O$). Relaxation occurs to an excited vibrational energy level in the ground electronic state and this is accompanied by an emission of light with decreased

frequency (υ_O-υ_m). With anti-Stokes scattering, excitation occurs from a molecule that is in an excited vibrational level of the ground state to a virtual state. This is followed by relaxation to a low level, resulting in an emission of light with the increased frequency (υ_O+υ_m). Both of these processes are displayed - **Figure 12**. The majority of Raman scattering is Stokes rather than anti-Stokes, resulting in the former producing more intense lines, which are more suitable for analytical purposes.

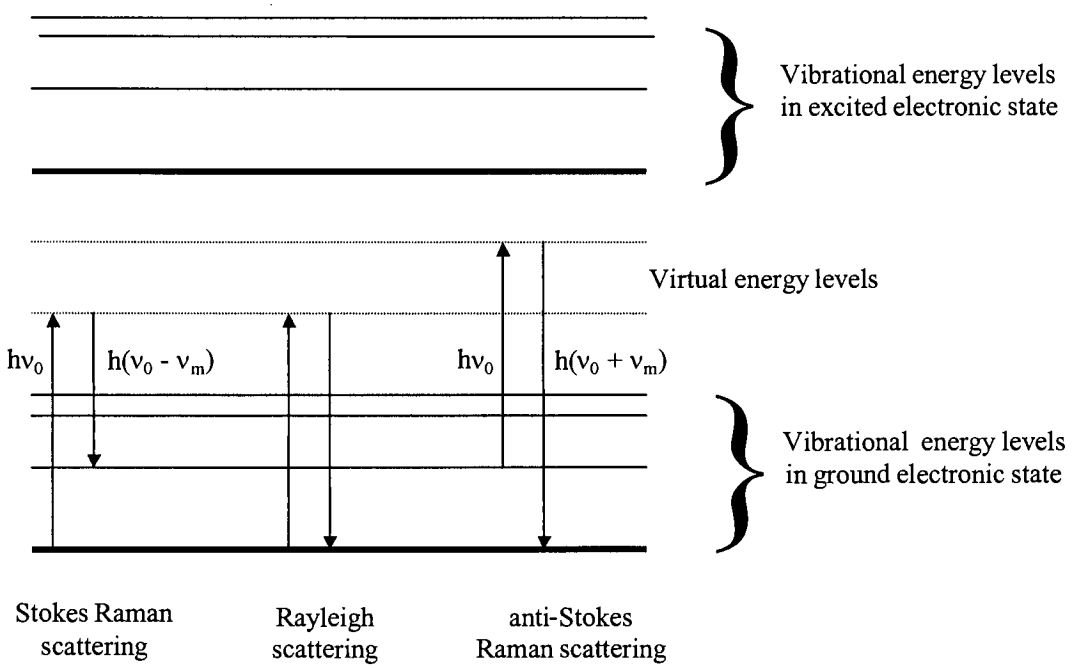


Figure 12: *Transitions involved in Rayleigh and Raman scattering.*

1.4.2 Resonance Raman Scattering

As previously mentioned, Raman spectroscopy can suffer from a lack of sensitivity. The inherent poor sensitivity can be addressed by using a laser excitation line which lies close to the absorption maximum of the analyte molecule.[120-122] The interaction of the incident photon with the analyte molecule promotes the molecule into an excited electronic state rather than a 'virtual state', as occurs in Raman scattering, before it returns to the ground state with the emission of the scattered photons. This results in an enhancement of approximately 10^3 to 10^4 over Raman scattering.

1.4.4 Surface Enhanced Raman Scattering

Surface enhanced Raman scattering (SERS) requires that the analyte is in close proximity to the suitably roughened surface of certain metals,of which gold and silver are the most widely used. In general, these roughened surfaces are generated on an electrode or by aggregating a colloidal suspension.[120] SERS is thought to be due to either or both of two general mechanisms: electromagnetic and/or charge-transfer (sometimes termed chemical) enhancement. Electromagnetic enhancement occurs when a surface plasmon is excited by the incident light, this in turn enhances the electromagnetic field of the incident light at the surface and any analyte adsorbed onto the surface experiences an enhancement of the its Raman scattering. Chemical enhancement has its basis in the generation of new electronic transitions from the metal surface to the adsorbate and vice versa, which allows charge –transfer excitations to and from the absorbate. The main analytical consequence of these processes is an enhancement of scattering of up to 10^6 in comparison to conventional Raman scattering. However, the magnitude of enhancement is dependent on the physical nature of the enhancing surface and on the orientation of the analyte at the surface.

1.4.5 Surface Enhanced Resonance Raman Scattering

SERRS combines molecular resonance with surface enhancement and the result is a technique which can provide sensitivity at the femto or even attomolar level.[120-122] This increase in sensitivity is due to enhancement factors of up to 10^{10} over conventional Raman scattering. When the frequency of the excitation laser is tuned to the molecular resonance of the analyte, the spectrum obtained is related to that of molecular resonance. This gives a molecularly specific signal from the analyte on the metal surface and allows positive identification and selective detection of analytes in the presence of nonresonant interferences. In addition, it should be possible to quantify the amount of one resonant analyte in the presence of another resonant species by deconvolution of the SERRS

spectra. In many cases, the interaction of the incident light with the chromophore reduces the dependence of the scattering on the orientation of the molecule to the surface[123]. In addition, energy transfer can occur from excited states of the molecule to the surface causing fluorescence quenching and enabling the use of a wide range of chromophores.[124] A graphical representation of SERRS is displayed - **Figure 13.**

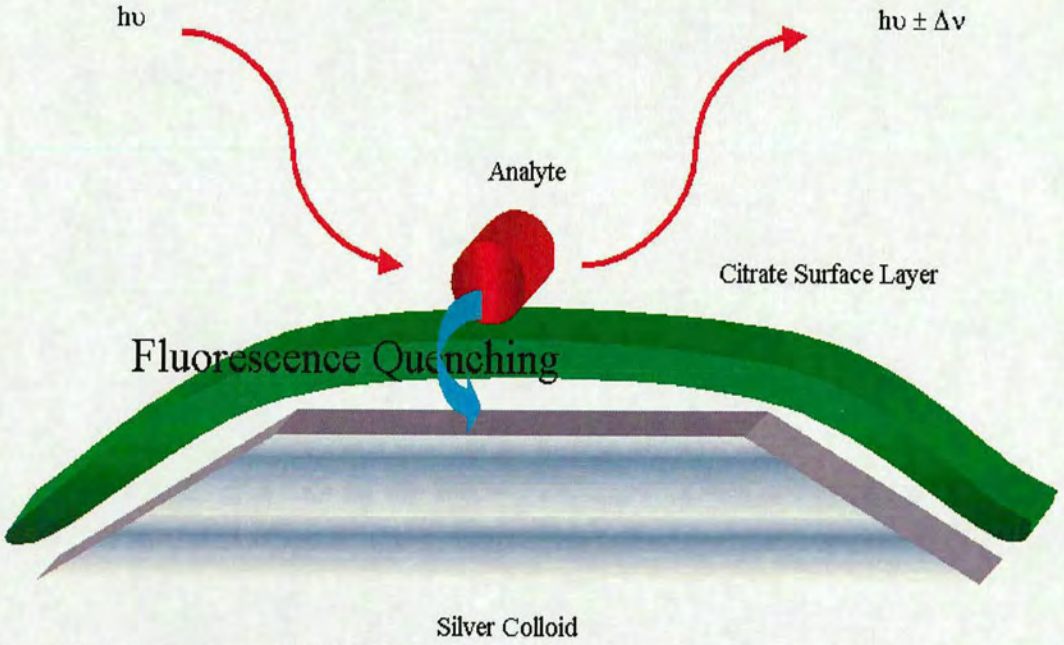


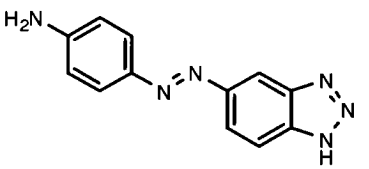
Figure 13: *Schematic of SERRS*

1.4.6 Quantitative analysis using SERRS

Quantitative analysis by SERRS has been reported[120] recently. However, it suffers from a lack of reliability and reproducibility due to the non-reproducible binding of the analyte to the colloidal surface. The non –uniform binding produces changes in the analytes spectrum. These changes are mainly caused by orientational changes and desorption of the analyte from the colloidal surface[125]. The use of monoazo-benzotriazole dyes have been shown[120-121] to circumvent these problems, due to the ability of benzotriazole to covalently attach to a metal surface. Benzotriazole compounds have been employed in a myriad of processes including corrosion inhibitors[126]; image

intensifies in photography, dyestuffs[127], chiral derivatisation agents[128] and as a synthetically useful moiety in organic chemistry[129].

Graham *et al.* reported[121] the synthesis of 4 monoazo-benzotriazole dyes **74-77** and the measurement of their SERRS spectra at concentrations ranging from $5x10^{-9}$M to 1 x 10^{-5}M. **76** was studied in more detail at concentrations between 5 x 10^{-9}M and 1 x 10^{-6}M and the signal was found to be proportional to concentration allowing quantitative analysis by SERRS.



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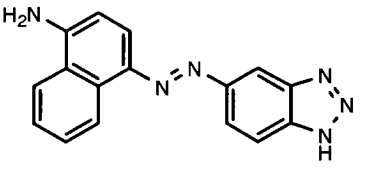

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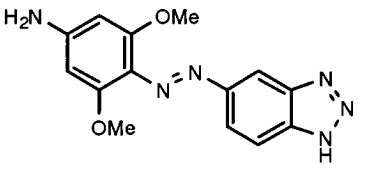

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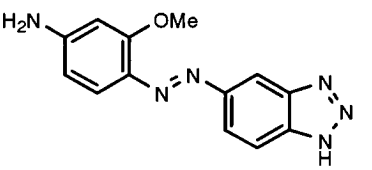
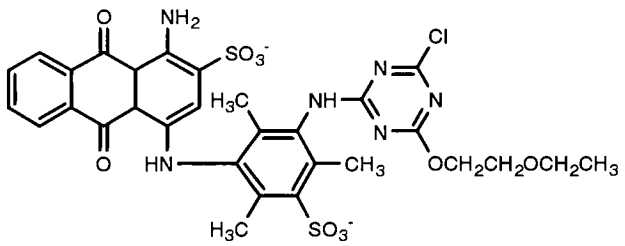


77

More extensive studies in the same laboratories[120, 125] reported the importance of colloid preparation, aggregation processes, linearity of response and the chemistry at the silver surface. Pre-aggregation of the colloid with poly (*L*-lysine) or spermine was found to give less SERRS enhancement than nitric acid or sodium chloride but the former aggregating agents were found to provide greater signal stability and therefore improve precision. At concentrations between 10^{-8}M and 10^{-12}M a proportional increase in intensity occurs with an increase in monoazo-benzotriazole dye concentration. At concentrations above 10^{-8}M no further increase in intensity was observed and this was attributed to complete monolayer coverage of the surface. Once monolayer coverage is achieved, further dye molecules do not contribute significantly to the SERRS signal obtained. The SERRS signals of monoazo-benzotriazole dyes **74-77** were compared[120] to those from a range of reactive dyes, including **78** and other similar structures.



78

In contrast, to the monazo-benzotriazole dyes, the reactive dyes did not display linearity of response over any concentration range and the spectrum obtained was different above and below 10^{-8}M. This indicated that the interactions of the two families of dyes with the silver surface were different. The monoazo-benzotriazole dyes are thought to displace the citrate layer, around the colloidal silver particles and bind strongly to the silver surface through the triazole ring - **Figure 13**. In contrast, the reactive dyes are expected to form polar interactions with the citrate layer. These polar interactions are thought to be much more susceptible to small changes in the chemical environment and thus give non-reproducible analyses.

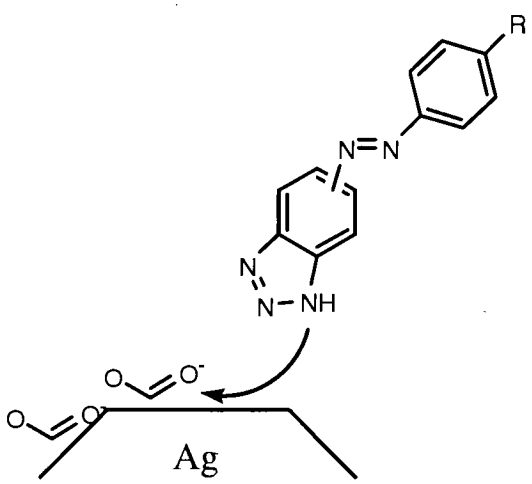
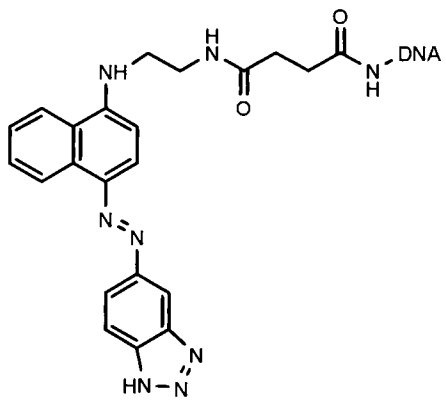


Figure 14: *Displacement of citrate layer and interaction of benzotriazole with silver surface*

1.4.7 Applications of SERRS

SERRS has been applied to a wide range of processes including the detection of labeled DNA (at 8 x 10^{-13}M),[130, 131] studies on mutant flavocytochrome b_2 (*L*-lactate:cytochrome c oxidoreductase from *Sacchromyces cerevisiae*)[132] and as an identification tool in column liquid chromatography[133]. In 2001, the first practical use of monoazo-benzotriazole as SERRS analytes was reported. Graham *et. al.*[130] applied a specifically designed benzotriazole dye **79** to the detection of poly-nucelic acid (PNA) and DNA. A 26-mer DNA oligomer and 8-mer PNA oligomer were labeled with **79** and the SERRS spectra obtained for both.



79

1.5 Aims of Project

With recent developments allowing a greater understanding of the experimental conditions that facilitate accurate and reproducible SERRS analysis it was envisaged that SERRS could be applied to the study of enzymatic reactions.

The SERRS activity of the benzotriazole dyes is dependant on interaction of the triazole ring with the silver surface[121] - **Figure 14.** In contrast, 1-substituted benzotriazole derivatives are postulated to be virtually SERRS inactive. Therefore by 'blocking' the triazole nitrogen of the monoazo-benzotriazole dye with an enzyme

cleavable moiety we can temporarily 'deactivate' the dye. Subsequent treatment of the 'blocked' dye with an enzyme would re-activate the dye and allow it to be detected by SERRS. This is represented in **Figure 15**.

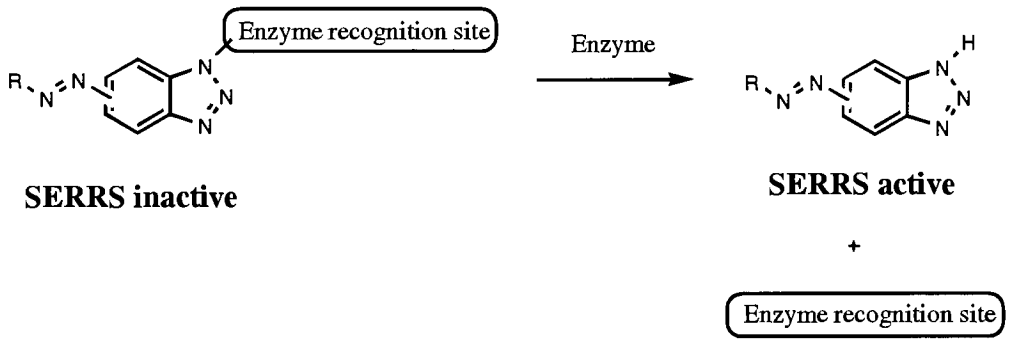


Figure 15: *Enzymatic conversion of 'blocked' SERRS dye to active SERRS dye*

It is envisaged that this can be extended to screening enzymatic resolutions by generating *pseudo*-enantiomers of the general form shown - **Figure 16** and treatment of these with enzymes. The initial rate of hydrolysis of the 2 *pseudo*-enantiomers can then be used to determine the enantioselectivity of the enzymatic reaction. Separation of the derivatised dyes is not required because SERRS can provide molecularly distinct spectra, thereby allowing the quantification of dye 1 in the presence of dye 2.

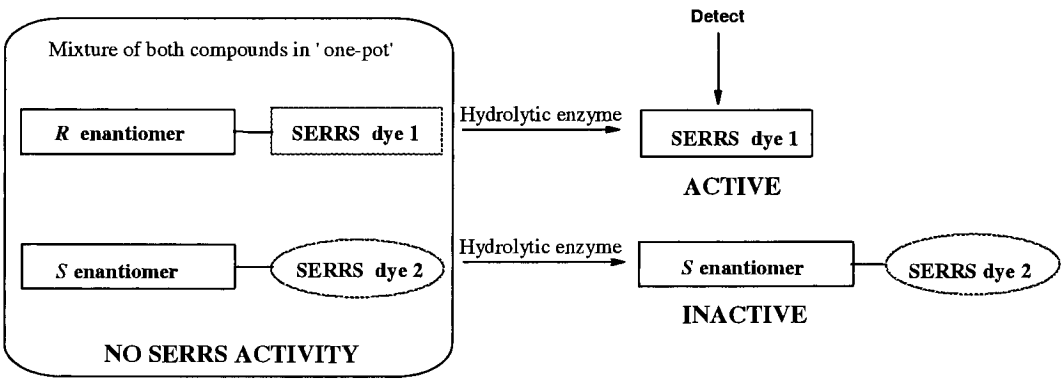
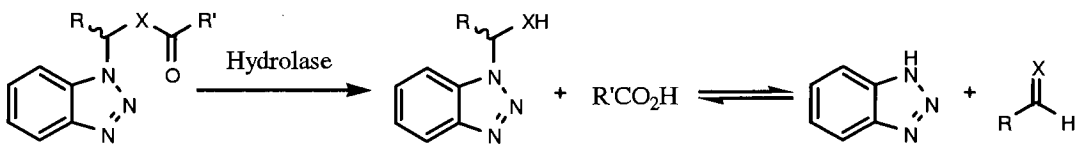


Figure 16: *Studying enzymatic resolutions using SERRS*

Further to this, is the application of SERRS to the screening of vast libraries of mutant enzymes generated by directed evolution. SERRS has already demonstrated its potential as an ultra-sensitive technique and developments are continuing in the design of high throughput measurements in 96-well plate format.

Therefore, the initial aims of this project are to synthesize compounds containing benzotriazole which are substrates for hydrolytic enzymes (*i.e* lipases/esterases but the system should also be applicable to proteases) *e.g.* **80.** Following enzymatic hydrolysis in aqueous media, the reaction product fragments to release benzotriazole **81** which can be detected – **Scheme 32**.



80 **81**

Where X= N or O

Scheme 32: *Fragmentation of enzymatic reaction products to release benzotriazole*

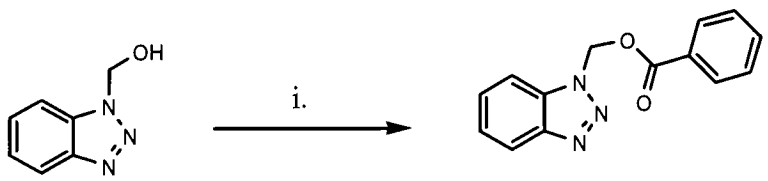
The chemistry developed for benzotriazole will then be applied to generate the *pseudo*-enantiomeric 'blocked' SERRS dyes. These 'blocked' SERRS dyes will then be analysed using SERRS, by our collaborators at the University of Strathclyde to determine if they are suitable substrates for enzymes and can be applied to determine the enantioselectivity of enzyme-catalysed reactions.

Chapter 2 - Results and Discussion I

2.1 Preliminary studies on benzotriazole

2.1.1 Reported syntheses of 1-(benzotriazol-1-yl)alkyl esters

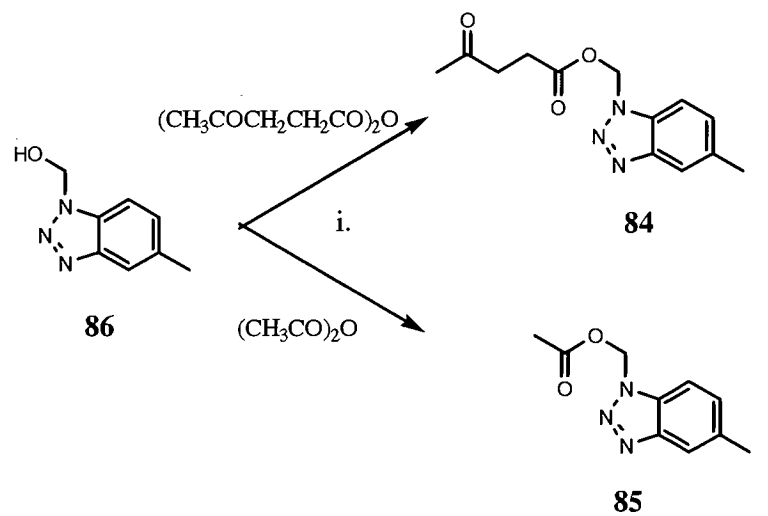
In 1957, Gaylord and Naughton[134] reported the synthesis of 1-benzoyloxymethyl-1*H*-benzotriazole **82** by direct treatment of 1-hydroxymethyl-1*H*-benzotriazole **83** with benzoyl chloride or benzoic anhydride in yields of only 27% and 24%, respectively – **Scheme 33**.



83 **82**

Scheme 33: *Reagents and conditions* i. PhCOCl or $(PhCO)_2O$, base

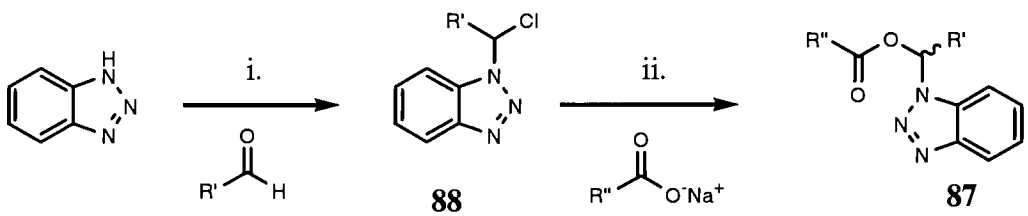
A similar approach was taken by Ono and Itoh[135] who prepared the levulinate and acetate esters **84** and **85** respectively, by the reaction of the appropriate anhydride with 5-methyl (1-hydroxymethyl)-1*H*-benzotriazole **86** - **Scheme 34**.



86 **84** **85**

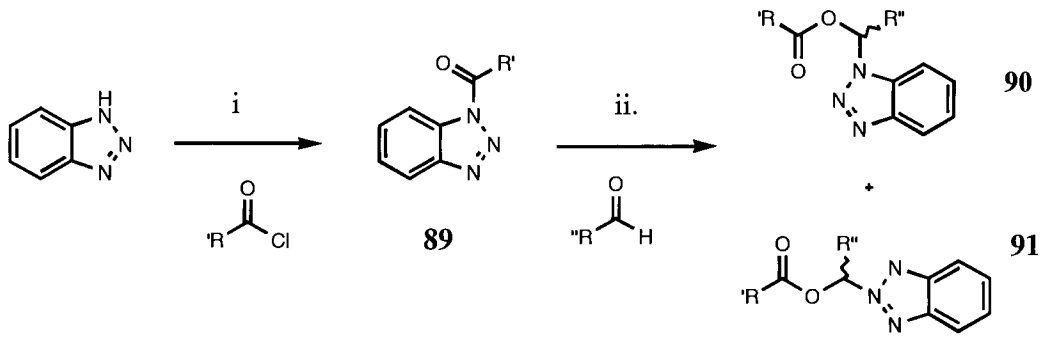
Scheme 34 *Reaction conditions* i. Pyridine (4 equivs.), DMAP (cat.), THF (anh.)

Further investigations into the preparation of 1-(benzotriazol-1yl)alkyl esters **87** led to the publication of two reports[136,137] by Katritzky *et al.*. The first, in 1991,[136] reported the synthesis of 1-(benzotriazol-1yl)alkyl esters, in yields of 71-90%, by the displacement of chlorine from 1-(1-chloroalkyl) benzotriazoles **88** with sodium carboxylates as shown - **Scheme 35**.



Scheme 35: *Reagents and conditions:* i. $SOCl_2$, $CHCl_3$, reflux, ii. DMSO, 50°C

In the later report, published in 1999, Katritzky *et al.*[137] synthesized benzotriazole esters by the reaction of *N*-acyl benzotriazoles **89** with either aliphatic or aromatic aldehydes in acetonitrile - **Scheme 36**. The reaction proceeded smoothly at room temperature with aliphatic aldehydes and electron deficient aromatic aldehydes but electron rich aromatic aldehydes required elevated temperatures. In general, the yields approached quantitative over two steps for the best case *e.g.* R' = C_6H_5 and R'' = C_2H_5. Most esters were obtained as the 1' isomer **90** however, some products contained small quantities (8-18%) of the 2'isomer **91**. The 2' isomer **91** seemed to be more readily formed when aliphatic aldehydes were employed, although this was greatly dependent on the reaction conditions used. The ratio of **90**:**91** ranged from 4:1 with acetonitrile to 10:1 with hexane. The implications of this will be discussed in more detail in **Section 3.3.**

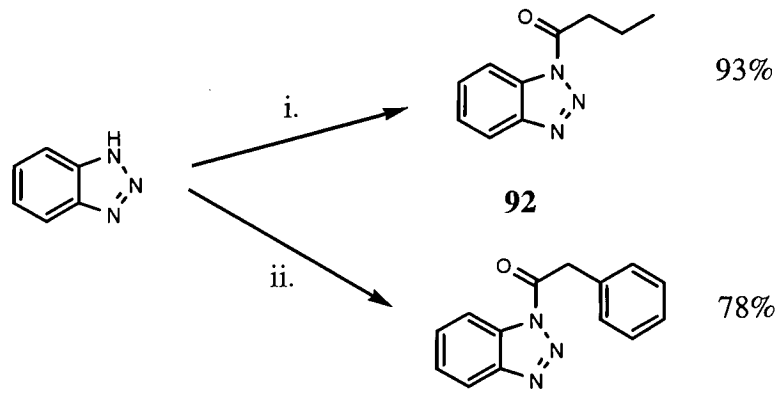


Scheme 36 *Reagents and conditions* i. Et_3N, DCM, ii. CH_3CN, base

2.1.2 Synthesis of 1-(benzotriazol-1-yl)alkyl esters from achiral *N* acyl benzotriazoles.

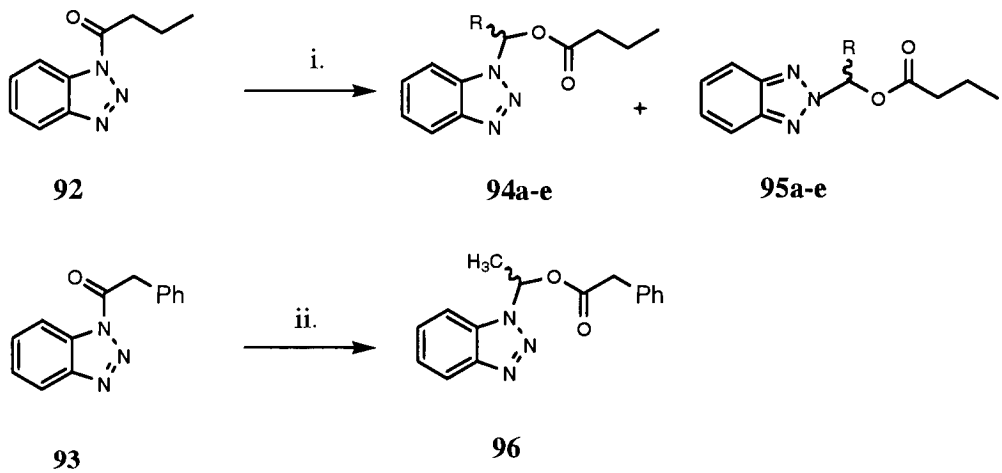
The synthetic route outlined - **Scheme 36** allows the generation of a range of 1-(benzotriazol-1-yl) alkyl esters **90** from readily synthesized *N*-acyl benzotriazole **89** simply by manipulating the aldehyde used. This synthetic route will form the basis of our initial investigations into these compounds. A range of these esters will be prepared and subjected to enzymatic hydrolysis to investigate if they are substrates for lipases/esterases and that they fragment, according to **Scheme 32**, to release benzotriazole into solution.

N-acyl benzotriazoles **92** and **93** were generated from the reaction of benzotriazole with the commercially available acid chlorides in anhydrous DCM with triethylamine - **Scheme 37.** The *N*-acyl benzotriazoles were obtained exclusively as the 1' isomer[137] in good to excellent yield. Butryl chloride was chosen because this will generate butyryl esters of benzotriazole and in general, butyrate esters are known substrates of lipases/esterases.[23] The *N*-acyl benzotriazoles were found to be relatively unstable at room temperature decomposing over a few weeks but could be stored for several months below 0°C.



Scheme 37: *Reagents and conditions* i. butryl chloride, DCM, Et_3N ii. phenylacetyl chloride, DCM, Et_3N.

N-acyl benzotriazole **92** was readily converted to the 1' esters **94a-e** and 2' esters **95a-e** by reacting with the appropriate aldehyde in CH_3CN with a catalytic amount of K_2CO_3 (15mol%) - **Scheme 38**. **93** was converted to the 1' ester **96** by reaction with propionaldehyde – the 2' ester was not isolated from the reaction mixture. The yields obtained are displayed - **Table 2**.



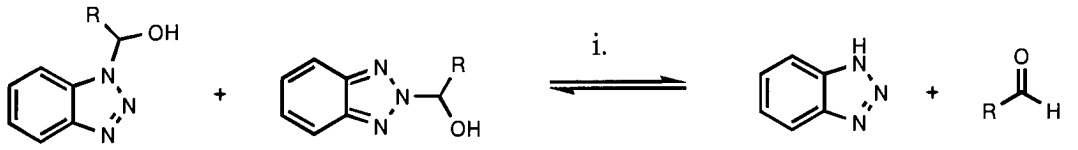
Scheme 38: *Reagents and conditions* i. RCHO, K_2CO_3, CH_3CN, RT, ii.CH_3CHO, K_2CO_3, CH_3CN

Compound	R'	Yield	Compound	Yield	Ratio 94:95
94a	H	68%	**95a**	3%	20.6:1
94b	CH_3	75%	**95b**	16%	4.7:1
94c	CH_2CH_3	68%	**95c**	15%	4.6:1
94d	$CH(CH_3)_2$	72%	**95d**	16%	4.7:1
94e	*p*-$NO_2C_6H_4$	62%	**95e**	*	-
96	CH_3	75%			

Table 2 *Yields and ratios of 94:95* * denotes: not isolated in pure form due to contamination with *p*-nitro benzaldehyde

The combined yields (**94+95**) obtained were slightly lower than those reported,[137] ranging from 82 to 88% compared to the quantitative conversions obtained by Katritzky and co-workers. The yield was lower despite the use of a much longer reaction time of 16 hours (the reactions were not complete in the 1-4 hours suggested by Katritzky). The slightly lower yield for **94a** may be due to the presence of H_2O and methanol, in the formaldehyde solution, which could breakdown the *N*-acyl benzotriazole. In addition, the ratio of **94a/95a** was markedly in favour of the 1' ester (~20:1) however, this was not as pronounced for **94b-d/95b-d.** The ratio of 1' (**94b-d**) to 2' (**95b-d**) were similar for these three esters and similar to that observed by Katritzky[137] (~4:1) under the same conditions.

Katritzky *et. al.*[138] investigated, by NMR, the effect increasing the steric bulk of the aldehyde had on the solution equilibrium of the benzotriazole adducts **97** and **98 – Scheme 39.** It was reported that the ratio of **97** to **98** was approximately 40:1 where R = H but remained constant, at ~ 10 to 13:1, across the series R = CH_3 / $(CH_3)_2CH$ / $(CH)_3C$. However, the proportion of adduct formed decreases in the order: CH_3CHO > $(CH_3)_2CHCHO$ > $(CH_3)_3CCHO$. These equilibrium studies support our observations for the ratio of **94a/95a** compared to **94b-d/95b-d.**



97 **98**

Scheme 39: *Conditions* i. deuterated solvent

2.1.3 Enzymatic hydrolysis of 1-(benzotriazol-1-yl)alkyl esters

With the benzotriazole esters in hand, their suitability as substrates for lipases and esterases had to be established. Small scale hydrolysis reactions (0.003mmol of each substrate) were performed using 9 lipases from Fluka Lipase Basic Kit and 10 esterases from Fluka Esterase Basic Kit – **Table 3** and **Table 4**. The substrates were incubated with the enzyme at room temperature in pH7 buffer for three hours and the amount of benzotriazole released determined by HPLC. All experimental details are given in **Chapter 5.**

Lipase	Conversion						
	94a	**94b**	**94c**	**94d**	**95d**	**94e**	**96**
Pseudomonas cepacia (PCL)	++	++	++	+	++	-	-
Porcine Pancreatic (PPL)	+++	+++	+++	+++	++++	+	++
Aspergillius niger	++	++	++	+	++	-	-
Mucor miehei	++	++	+	+	++	-	-
Pseudomonas fluorescens (PFL)	++	++	++	+	++	-	-
Rhizopus niveus	+	+	+	+	+	-	-
Rhizopus arrhizus	+	+	+	+	++	-	-
Candida antarctica (CAL)	100	100	100	100	100	++	+++
Candida cylindracea (CCL)	++++	++++	100	++++	100	++	++
Control	-	-	-	-	-	-	-

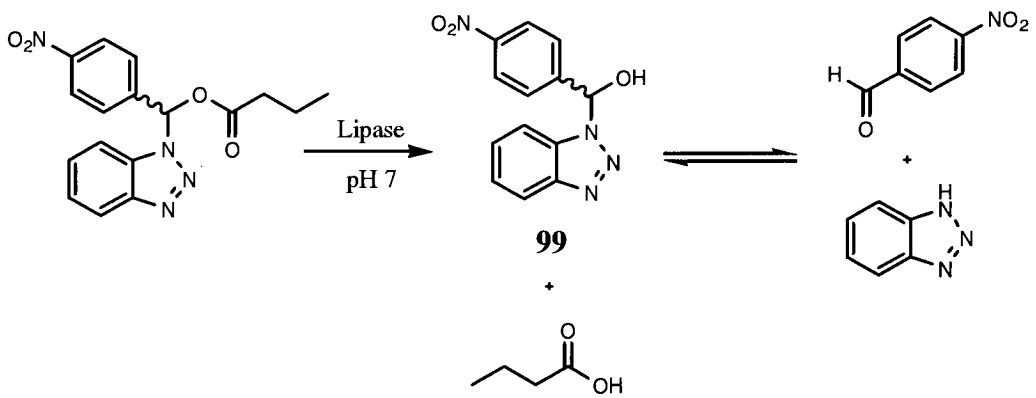
Table 3: + (0-25%), ++ (26-50%), +++ (51-75%), ++++ (76-99%).

Esterase	Conversion					
	94a	**94b**	**94c**	**94d**	**95d**	**94e**
Candida lipolytica	++	++	+	+	+	+
Horse Liver	++	++	+	++	++	+
Hog Liver	100	100	100	++++	++++	++++
Control	-	-	-	-	-	

Table 4: + (0-25%), ++ (26-50%), +++ (51-75%), ++++ (76-99%).

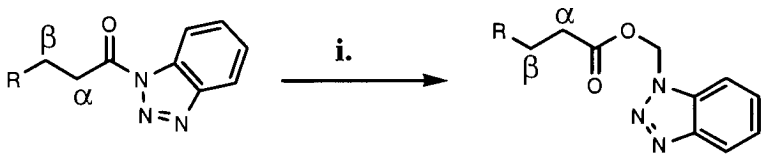
The qualitative results displayed in **Table 3** indicate that all of the benzotriazole esters were hydrolysed, at least to some extent, by at least one lipase from the kit. Encouragingly, both the 1' isomer **94d** and 2'isomer **95d** were hydrolysed by all nine lipases.

With **94e**, where R = *p*-$NO_2C_6H_4$ only CAL, CCL and PPL display activity and much less hydrolysis was detected compared to the esters generated from aliphatic aldehydes. In addition, to benzotriazole, *p*-nitrobenzaldehyde was detected by HPLC for these substrates indicating that the hemiaminal product **99** fragmented further - **Scheme 40**. With ester **96** only CAL, CCL and PPL displayed activity towards this ester



Scheme 40: *Fragmentation of* **99** *to p-nitrobenzaldehyde and benzotrizole*

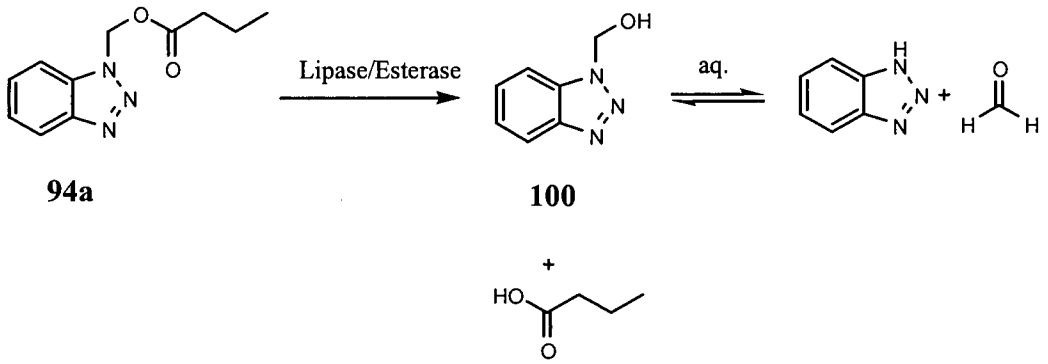
The results displayed in **Table 4** indicate that of the 10 esterases tested only *Candida lipolytica*, horse liver and hog liver esterase exhibit activity towards the benzotriazole esters. Again, both the 1' isomer **94d** and the 2' isomer **95d** were hydrolysed to the same degree. Ester **96** was not subjected to hydrolysis by the esterases. Overall, the benzotriazole esters (**94a-e**, **95d** and **96**) seem to be substrates for lipases and esterases. At this point, it was decided to concentrate on esters generated from formaldehyde *i.e.* **94a** since these esters are generated from primary alcohols and as such are achiral. Esters of this type allow introduction of a chiral centre in the acyl portion (either α, β or more remote from the carbonyl) by simply starting from chiral *N*-acyl benzotriazoles – **Scheme 41**.



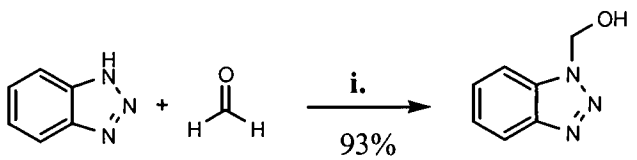
Scheme 41: *Reagents and conditions:* i. CH_2O, base

This would be crucial when we attempt to synthesize the blocked SERRS dyes since we require substrates with only one chiral centre. Esters **94b-e** and **96** contain a racemic secondary alcohol that cannot be synthesized in enantiomerically pure form using this reaction. However, these esters are good model compounds to study the action of lipases on substrates bound to combinatorial chemistry resins – this is discussed in **Section 2.1.11.**

At this point it was still unclear, whether the 1-hydroxymethylbenzotriazole **100** generated from the hydrolysis of the ester **94a** fragmented further to release benzotriazole – **Scheme 42**. Previously, Ono and Itoh[135] had utilised the aqueous instability of 1-hydroxymethylbenzotriazoles to generate the free benzotriazole and Katritzky *et al.*[138] had reported the solution instability of other hemiaminals generated from aliphatic aldehydes. In order to investigate this, 1-hydroxymethylbenzotriazole **100**, was synthesized from formaldehyde and benzotriazole according to the method reported by Hall *et. al.*[139]. – **Scheme 43.**



Scheme 42: *Aqueous fragmentation of 1-hydroxymethylbenzotriazole.*



Scheme 43: *Reaction conditions: i.* acetic acid/H_2O.

1-Hydroxymethylbenzotriazole **100** was obtained as a white precipitate in excellent yield (93%). ^{1}H NMR and ^{13}C NMR indicated that the 1-hydroxymethylbenzotriazole was formed exclusively as the 1' isomer. Using the reverse phase HPLC method used to study the enzymatic cleavage reactions, **100** was found to co-elute with benzotriazole. Further method development generated an isocratic method that resolved benzotriazole from **100**. HPLC analysis of an acetonitrile solution of **100** gave two peaks – one at the retention time of benzotriazole (<5 area%, 3.3 mins) and the other at 3.1 mins which was assigned as the 1-hydroxymethylbenzotriazole **100 – Figure 17**. Spiking of this solution with benzotriazole increased the size of the peak at 3.3mins. Analysis of an aqueous buffered solution (pH7) of **100** gave essentially one peak at the retention time of benzotriazole (~1% of **100** was detected) - **Figure 18**. This confirmed that **100** was unstable in buffer solution although a small amount of on-column degradation of **100** to benzotriazole had been detected.

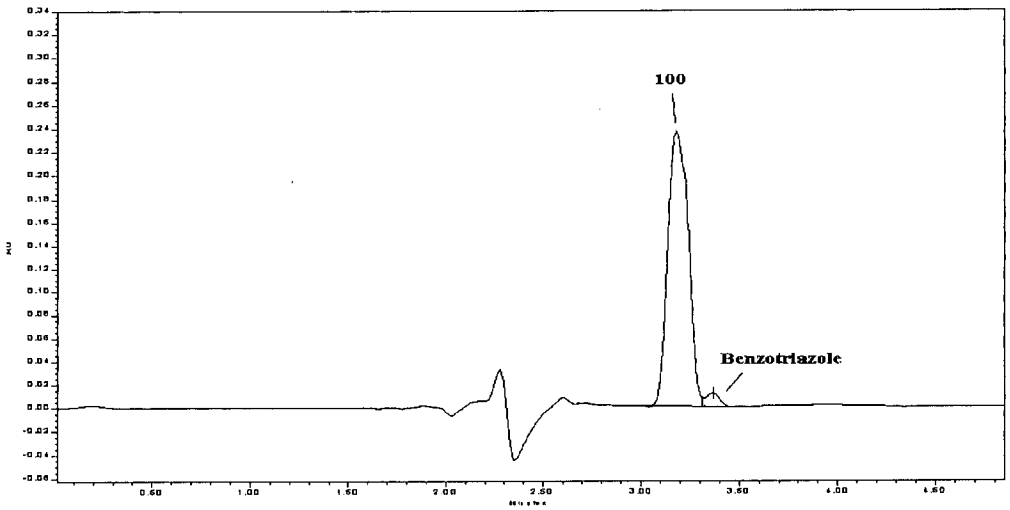


Figure 17: *HPLC analysis of* **100** *in* CH_3CN

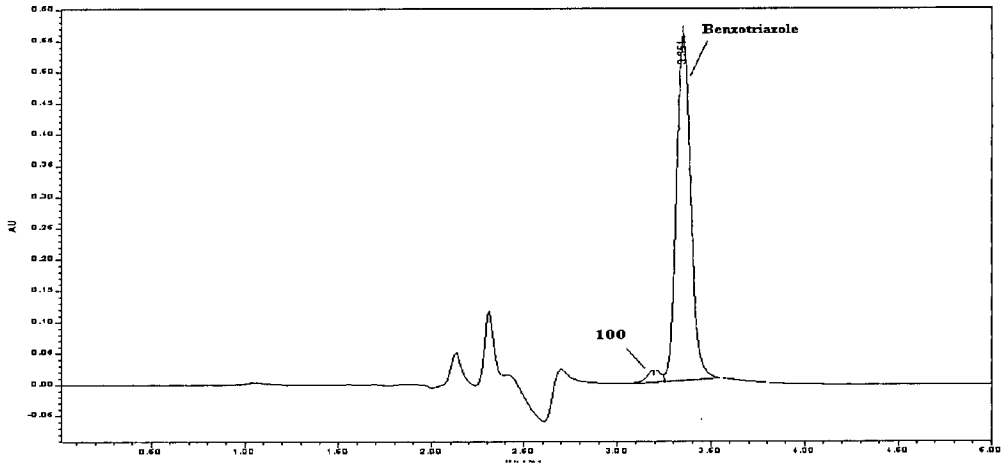
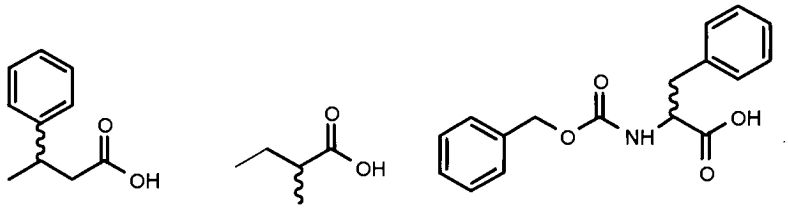


Figure 18: *HPLC analysis of* **100** *in aqueous buffer*

2.1.4 Preparation of 1-(benzotriazol-1-yl)alkyl esters from chiral acyl-benzotriazoles

For SERRS analysis to be successful, it was necessary to generate enantiomerically pure *pseudo*-enantiomers of the blocked SERRS dyes. Following the successful synthesis of ester **94a**, it was decided to investigate benzotriazole esters containing chiral acyl moieties. Three different acids were chosen: 3-phenylbutyric acid **101,** 2-methylbutyric acid **102** and Z-protected phenylalanine **103**. These acids would

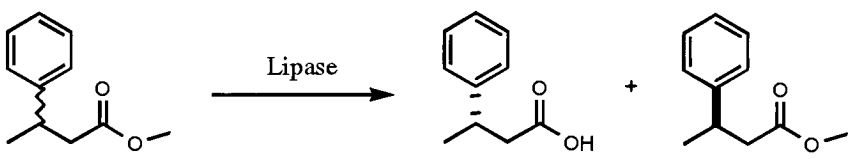
allow investigation of the ease with which benzotriazole esters with both α and β chiral centres could be prepared. Esters generated from **103** should be substrates for α–chymotrypsin, a serine protease, known[2, 144] to hydrolyse protected aromatic amino acid esters and amides.



101 **102** **103**

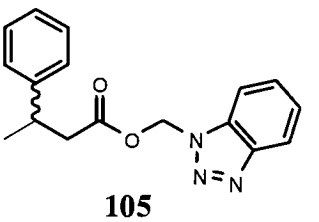
2.1.5 Synthesis of 3-phenylbutyric acid benzotriazol-1yl-methyl ester

Investigations began with (*S*)-3-Phenylbutyric acid **101** because it was thought to be the least likely to undergo racemisation of the chiral centre and both isomers were commercially available. In addition, Reeve et al[138] reported the preparative scale enzymatic hydrolysis of 3-phenylbutyric acid methyl ester **104** with *E* values ranging from 2 to >153 using porcine liver esterase (Chirazyme E1 and E2) and *Chromobacterium viscosum* lipase, respectively – **Scheme 44**. This should allow us to compare the *E* values reported for the methyl ester **104** with those determined for the benzotriazole ester **105**.



104 (*S*) **-101** (*R*)**-104**

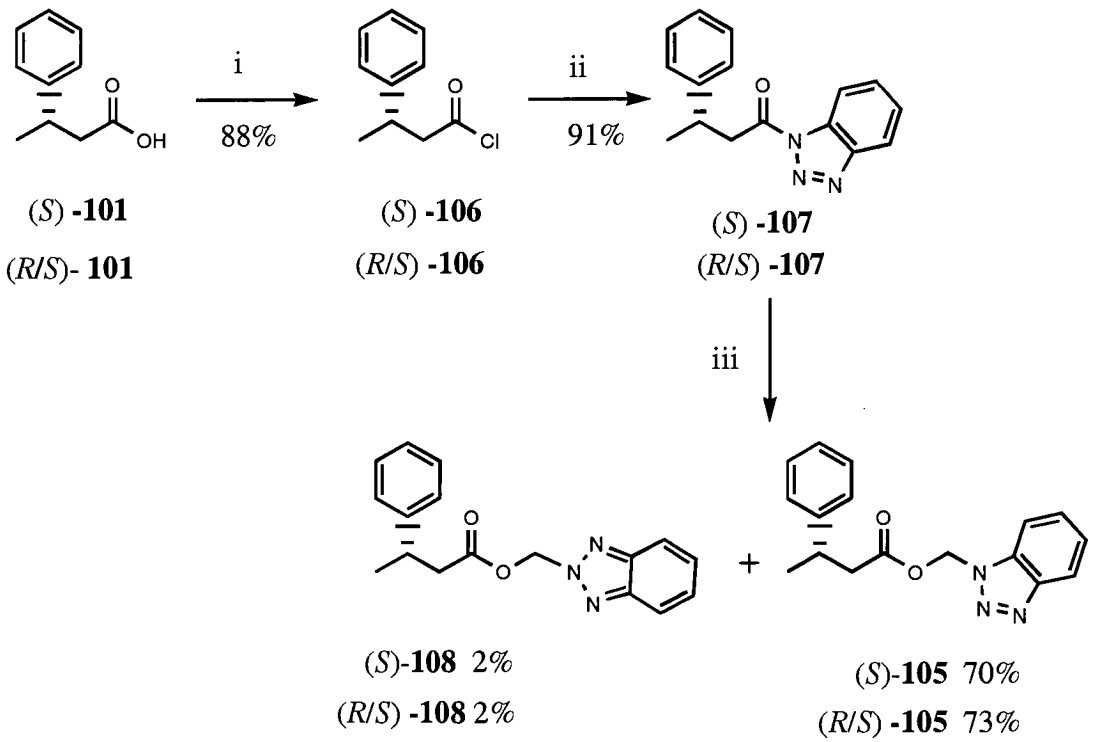
Scheme 44: *Conditions* i. pH 7.8 phosphate buffer



105

Starting from commercially available (*S*)-**101,** the acid chloride (*S*)-**106** was synthesized in 88% yield by treatment with thionyl chloride – **Scheme 45**. Subsequent formation of the *N*-acyl benzotriazole derivative (*S*)-**107,** exclusively as the 1' isomer, was also achieved in excellent yield. The esters (*S*)-**105** and (*S*)-**108** were isolated in 70% and 1.6% yield respectively by treatment of (*S*)-**107** with formaldehyde and 25mol% K_2CO_3. This ratio of (*S*)-**105** to (*S*)-**108** ester was similar to that which had been observed for **94a** to **95a**. The ^1H NMR of **105** and **108** gave the $C\underline{H}_2O$ as AB doublets with J values of 11.2 and 9.9Hz respectively. The ^{13}C NMR signal for the $C\underline{H}_2O$ was shifted from 67.3 p.p.m. in **105** to 74.9 p.p.m. in **108**.

The enantiomeric purity of **105** was determined by HPLC to be >99% e.e. which is in excellent agreement with the starting acid (99% e.e. determined by HPLC) indicating that no racemisation of the chiral centre had occurred during the synthesis – **Figure 19**. The above reactions were repeated with (+/-)-**101** to give (+/-)-**106, 107, 105** and **108**. (+/-)-**105** was used as an HPLC standard to measure the enantiomeric purity of (*S*)-**105** and will be used for the enzymatic resolution studies – **Section 2.2**.



Scheme 45: *Reactions and conditions* i. $SOCl_2$, ii. benzotriazole, DCM, Et_3N, 0°C, iii. formaldehyde, CH_3CN, K_2CO_3.

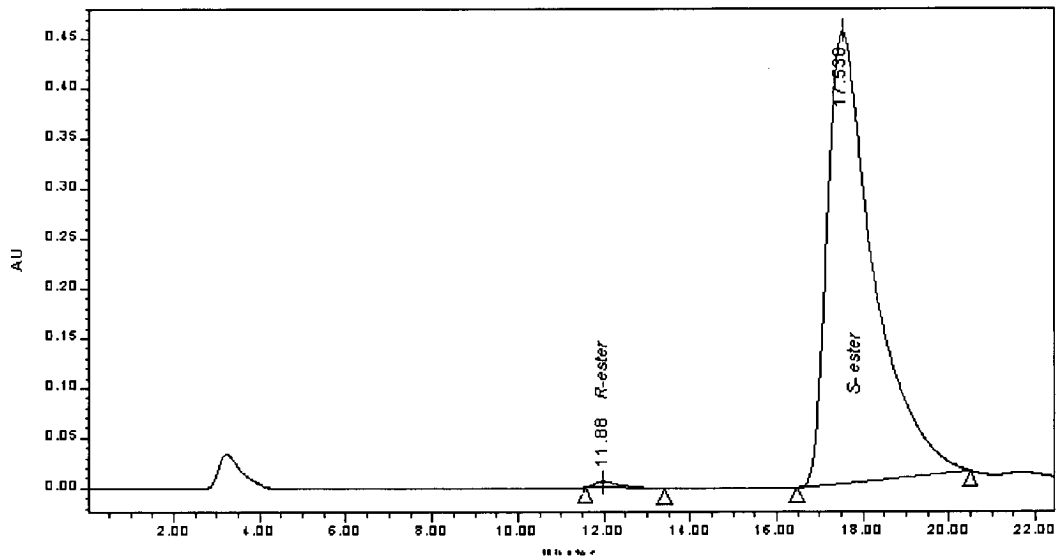
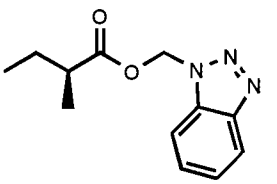


Figure 19: *Enantiomeric purity of (S)-**105***

2.1.6 Preparation of benzotriazole esters from acids with an α-chiral centre

Following the successful synthesis of (*S*)-**105** attention was turned to the synthesis of (*S*)-**111**.



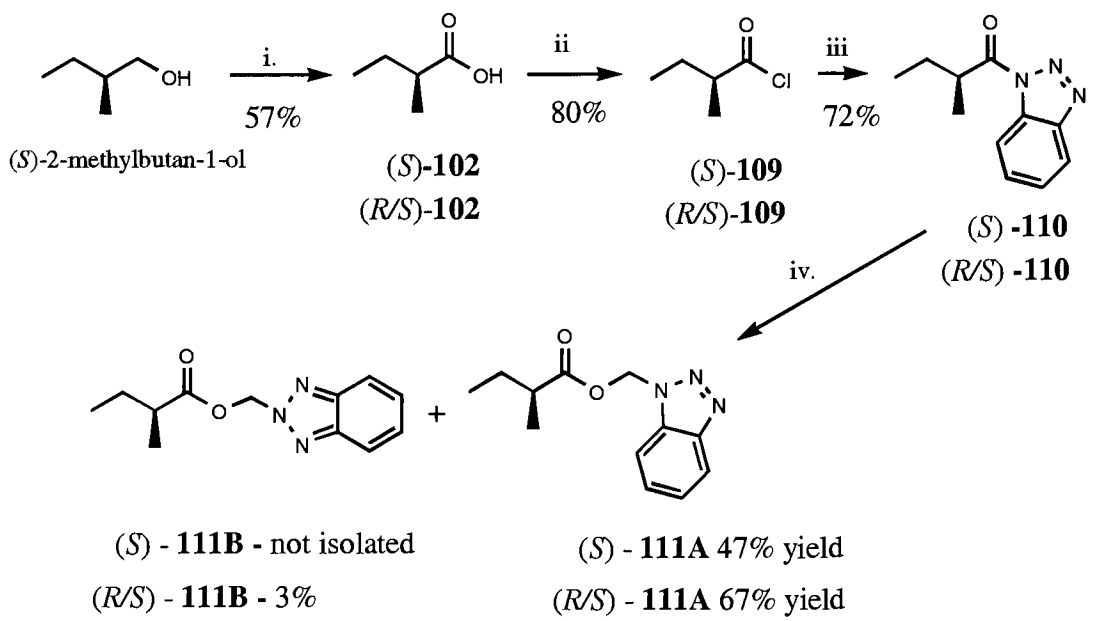
(*S*)-**111**

Acids with an α–chiral centre will be more prone to racemization due to the increased acidity of the proton at the chiral centre and as a result the synthetic route used should minimise the use of base. However, from preliminary investigations into the synthesis of the blocked SERRS dyes, which were carried out in tandem, it appeared that routes to these via the *N*-acyl derivative would be most successful – discussed in more detail in **Chapter 3**. With this in mind initial investigations focused on a route similar to that used for (*S*)-3-phenylbutyric acid (*i.e.* **Scheme 45**).

2.1.7 Synthesis of 2-methyl butyric acid benzotriazole-1-yl methyl ester

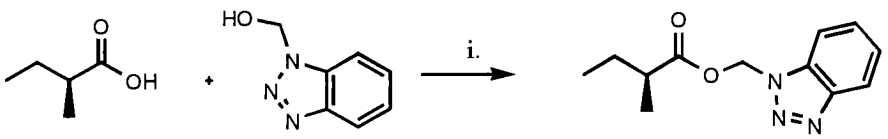
Starting from commercially available (*S*)-2-methylbutan-1-ol (>98% e.e.) - **Scheme 46**, (*S*)-**102** was synthesized in 57% yield by permanganate oxidation according to the procedure detailed by Begley *et. al.*.[141] The literature yield was moderate (55%) and attempts to improve the yield of (*S*)-**102** using Jones oxidation were unsuccessful, with only a 38% yield obtained. Conversion to the acid chloride (*S*)-**109** using thionyl chloride proceeded in good yield again as described by Begley *et. al.*.[141] Formation of the *N*-acyl benzotriazole (*S*)-**110,** as the 1' isomer, by reaction of (*S*)-**109** with benzotriazole proceeded in good yield. Conversion to the ester (*S*)-**111A** was achieved by treatment of (*S*)-**110** with formaldehyde in CH_3CN with 15mol% potassium carbonate. The ester (*S*)-**111A** was isolated as a thick clear oil which solidified on freezing in 47% yield. Due to the small scale of the reaction a sample of the 2' isomer (*S*)-**111B** was not isolated.

Steps ii-iv in **Scheme 46** were repeated with commercially available (*R/S*)-2-methylbutyric acid to give (*R/S*)-**109, 110, 111A** in comparable yields to the optically pure compound. (*R/S*)-**111B** was also isolated in 3% yield.



Scheme 46: *Reagents and conditions*: i. $KMnO_4$, Na_2CO_3, 0°c, ii. $SOCl_2$, iii. benzotriazole, DCM, Et_3N, 0°c, iv. formaldehyde, CH_3CN, K_2CO_3.

The enantiomeric purity of ester (*S*)-**111A** was determined by normal phase HPLC and found to be 75% e.e. This indicated that a substantial amount of racemisation had occurred during preparation. Treatment of (*S*)-**111A** with Et_3N or K_2CO_3 in CH_3CN did not alter the enantiomeric purity proving that the ester was not sensitive to base. Attempts to determine the enantiomeric purity of (*S*)-**110** by chiral HPLC, which was the only intermediate in the synthetic route with a chromophore, were unsuccessful. The other intermediates in the synthetic route all gave comparable optical rotation values to those previously reported. Other routes to (*S*)-**111A** were investigated to determine if the racemization could be minimized and reduce the number of synthetic steps - **Scheme 47**. A variety of coupling reagents and conditions were investigated.



(*S*) **-102** **100** (*S*) **-111A**

Scheme 47: *Reagents and conditions*: i. See **Table 5**

The coupling reactions were carried out on a small scale and then analysed by HPLC to determine the yield of (*S*)-**111A.** Any reactions that appeared promising, were scaled up and (*S*)-**111A** isolated. The results are displayed - **Table 5**.

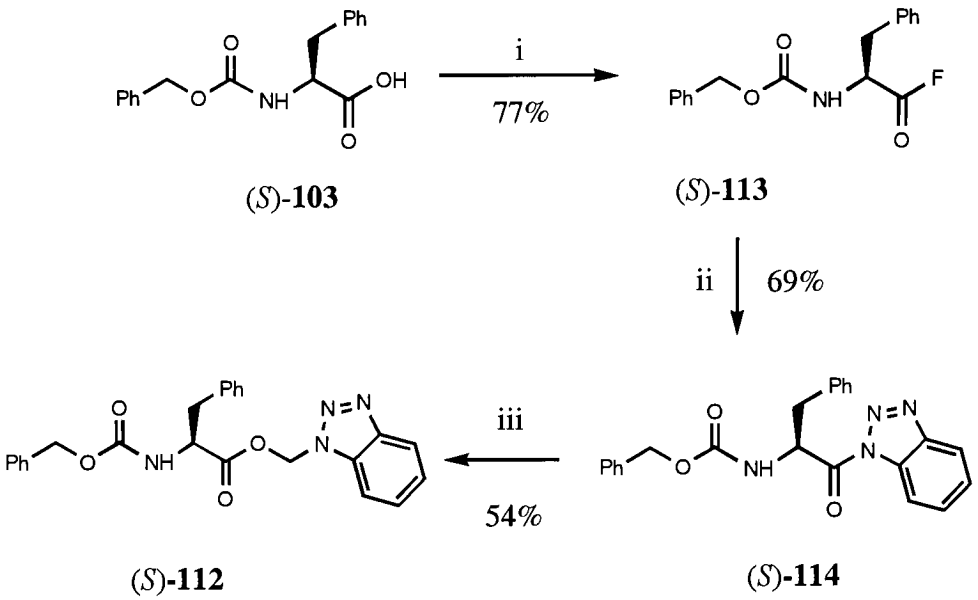
Entry	Conditions (i)	Area % **111A**	Isolated yield **111A**	% e.e (*S*)-**111A**
1	EEDQ, DCM	6.5%	-	-
2	DCC, DIPEA, DCM	36.6%	-	-
3	DCC, DMAP, DCM	37.0%	-	-
4	PPh_3, DEAD, THF	72.5%	78%	>98%
5	PPh_3, DEAD, DMF	-	43%	>98%
6	PPh_3 (resin bound), DEAD, DMF	N/D	N/D	N/D

Table 5: *Coupling of **100** with (S)-**102***

Note: Entry 5 from **Table 5** was performed because it was known that the monoazo- benzotriazole dyes are sparingly soluble in most organic solvents but readily soluble in DMF and the use of this solvent maybe necessary in the synthesis of the 'blocked' SERRS dyes. The synthesis of the blocked SERRS dyes is discussed in more detail in **Chapter 3**. As can be seen from **Table 5,** Mitsunobu conditions in THF gave a good yield of (*S*)-**111A** with an excellent e.e. This allows the preparation of the benzotriazole esters in one step from the parent acid.

2.1.8 Synthesis of benzotriazole esters from protected amino acids

For the synthesis of (*S*)-**112** the acid fluoride of Z-phenyalanine **113** was used in preference to the acid chloride because it is known[142] that in general the former are more stable to hydrolysis and Z-amino acid chlorides are unstable upon treatment with base. Z-*L*-phenylalanine **103** was converted to the acid fluoride (*S*)-**113** by treatment with cyanuric fluoride using the method described by Bertho et al[143] in good yield and with excellent enantiomeric purity – **Scheme 48**. (*S*)-**113** was converted to the *N*-acylbenzotriazole (*S*)-**114** by reaction with benzotriazole in the presence of base and then converted to the ester (*S*)-**112** by treatment with formaldehyde.

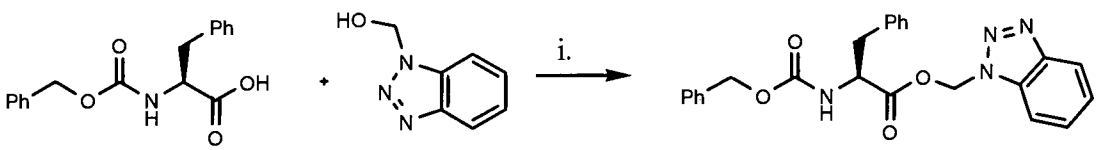


(*S*)-**103** (*S*)-**113** (*S*)-**112** (*S*)-**114**

Scheme 48: *Reagents and conditions* i.) cyanuric fluoride, pyridine, DCM, ii.) benzotriazole, Et_3N, DCM, iii.) formaldehyde, CH_3CN, K_2CO_3.

The enantiomeric purity of (*S*)-**112** was determined, by normal phase Chiral HPLC, as 90% e.e. after comparison with a standard of (*R/S*)-**112.** (*R/S*)-**112** had been generated in 24% yield from the coupling of 1-hydroxymethylbenzotriazole with (*R/S*)-**103** using EDCI. In light of the racemization detected in (*S*)-**112** synthesized via the acid fluoride, the optically pure form of (*S*)-**112** was synthesized with >99% e.e. using EDCI

and HOBt – **Scheme 49**. Although the yield of this single step was relatively modest the yield is greater than the overall yield achieved for synthesis of (*S*)-**112** from the acid fluoride. No optimisation of this reaction was performed.

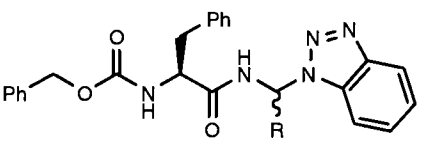


(*S*) - **103** **100** (*S*)-**112**

Scheme 49: *Reaction conditions* i. EDCI, HOBt, DIPEA, DCM

2.1.9 Preparation of *N*-[1-(benzotriazol-1-yl)alkyl] amides

Following the successful synthesis of (*S*)-**112** it was decided to extend our synthetic methodology to amides. The general structure **115** was proposed.

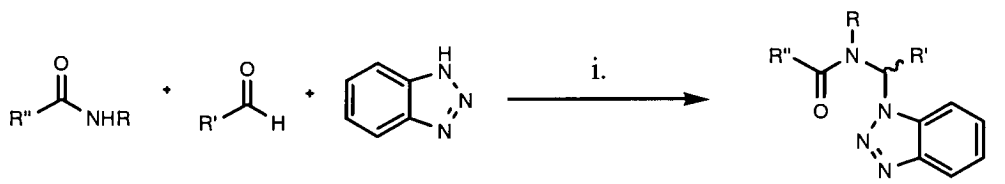


115

Compounds of type **115** should be substrates for α–chymotrypsin, a serine protease, which in nature[2, 144] acts as a protein endopeptidase and catalyses the hydrolysis of non terminal peptide bonds that are adjacent to a phenylalanine, tyrosine or tryptophan residue. It also accepts a broad range of aromatic amino acid amides, esters and other carboxylic acid derivatives.

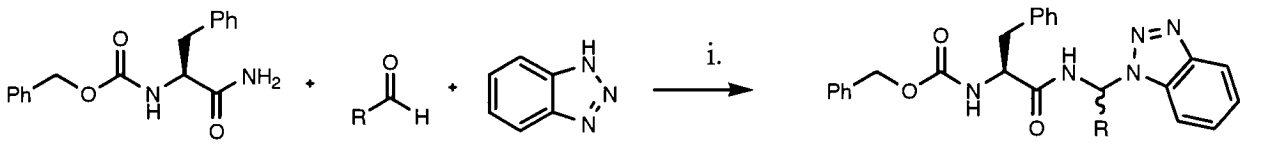
Katritzky and co-workers published several reports[129, 145-148] on the synthesis of compounds similar to **115**. The reaction occurs in moderate to good yields from the condensation of an amide, aldehyde and benzotriazole in refluxing toluene under Dean Stark conditions – **Scheme 50**. The reaction is fairly general for most primary (including protected amino acid amides, as in our case) and secondary amides (*i.e.* R≠H). The yield

of adduct is generally improved by the addition of a catalytic amount of *p*-toluenesulphonic acid to the reaction mixture.



Scheme 50: *Reagents and conditions* i. toluene (cat. TsOH) or acetic acid, reflux, Dean Stark apparatus

This reaction was explored with commercially available Z-*L*-phenylalanine amide **116**, benzotriazole and a variety of aldehydes to generate two benzotriazole adducts **117** and **118 - Scheme 51.**



116 **117-118**

Scheme 51: *Reagents and conditions* i. toluene, cat. TsOH, reflux, Dean Stark

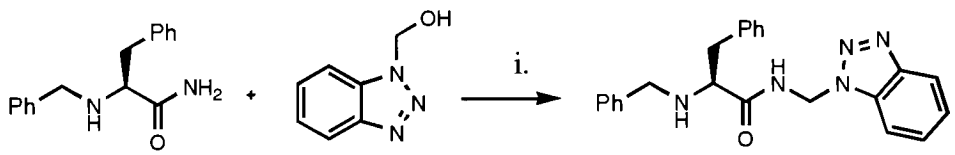
Initial results from this reaction were disappointing. The use of aromatic aldehydes resulted in complex mixtures of products and starting materials, making isolation of the desired adduct very difficult. In contrast, the use of *iso*butyraldehyde or formaldehyde resulted in the isolation of the desired product, albeit in low yields - **Table 6.**

Adduct	R	Yield	Ratio of Diastereomers	
			HPLC	NMR
117	$CH(CH_3)_2$	44%	1:1	1:1
118	H	24% (33%)*	*	*

Table 6: *Preparation of adducts* **117** *and* **118**. * denotes see text.

The benzotriazole adducts **117** and **118** were obtained as the 1' isomers, confirmed by comparison with the ^{1}H NMR of other 1-substituted benzotriazole compounds. ^{1} H NMR and reverse phase HPLC analysis indicated that **117** was formed as a 1:1 mixture of diastereomers. At this point, it was unclear whether the diastereomers of **117** would be hydrolysed with equal rates by α–chymotrypsin – this will be discussed in **Section 2.2.4.**

The maximum yield (44%) of **117** was achieved after careful distillation of the *iso*-butyraldehyde under nitrogen and the introduction of a stirring step prior to refluxing. The yield of adduct **118** was increased slightly from 24% to 33% by refluxing 1-hydroxymethylbenzotriazole **100** with **116** in glacial acetic acid – **Scheme 52**. The enantiomeric purity of **118** was determined as >99% e.e. using normal phase chiral HPLC.



116 **100** **118**

Scheme 51: *Reagents and conditions* i. acetic acid, reflux.

2.1.10 Summary

We have designed synthetic routes to *N*-substituted benzotriazole esters and amides that contain optically active acids. Esters generated from acids with a β–chiral

centre could be synthesised in excellent enantiomeric purity from the *N*-acyl benzotriazole. With protected amino acids and α chiral acids, the chiral centre was found to be more prone to racemisation. In these cases, the esters were synthesized in optically pure form using either EDCI and/or Mitsunobu conditions. Access to the amides was achieved in a one-pot reaction in moderate yield.

2.1.11 Future Work

In light of the racemisation observed in the synthesis of (*S*)-**111 (A+B)** and (*S*)-**112** from *N*-acyl benzotriazoles, a variety of bases could be used to determine if this could be reduced. In hindsight the use of pyridine instead of triethylamine, in the acylation step, may have avoided this racemisation. Further optimization studies on the synthesis of 1-(benzotriazole-1-yl) alkyl esters have been performed and will be discussed in **Chapter 3, Section 3.3**.

Further optimization studies on the synthesis of 1-(benzotriazol-1-yl) alkyl amides may result in improved yields. However, significant development work has already been performed[149] in our laboratory, on a similar reaction, and yields remained inconsistent.

As mentioned previously, esters **94b-e**, **95b-d, 96** and amide **117** may be useful model compounds to investigate the design of enzyme cleavable linkers[150] – **Figure 20**. Potentially these could be synthesized in three different ways, either linking to the resin through benzotriazole, or via an aldehyde or amide. The synthesis of resin bound benzotriazole[151-153] has been reported in the literature and compounds similar to linker type 1 are currently being investigated,[154] in our laboratory, for the attachment of peptide aldehydes to solid supports. In addition, linker type 1 could be used in enzymatic resolutions of resin bound esters. The un-hydrolysed ester would remain bound to the solid support allowing simple separation. A similar structure to linker type **3** has been developed as a penicillin amidase cleavable linker for alcohols[155]

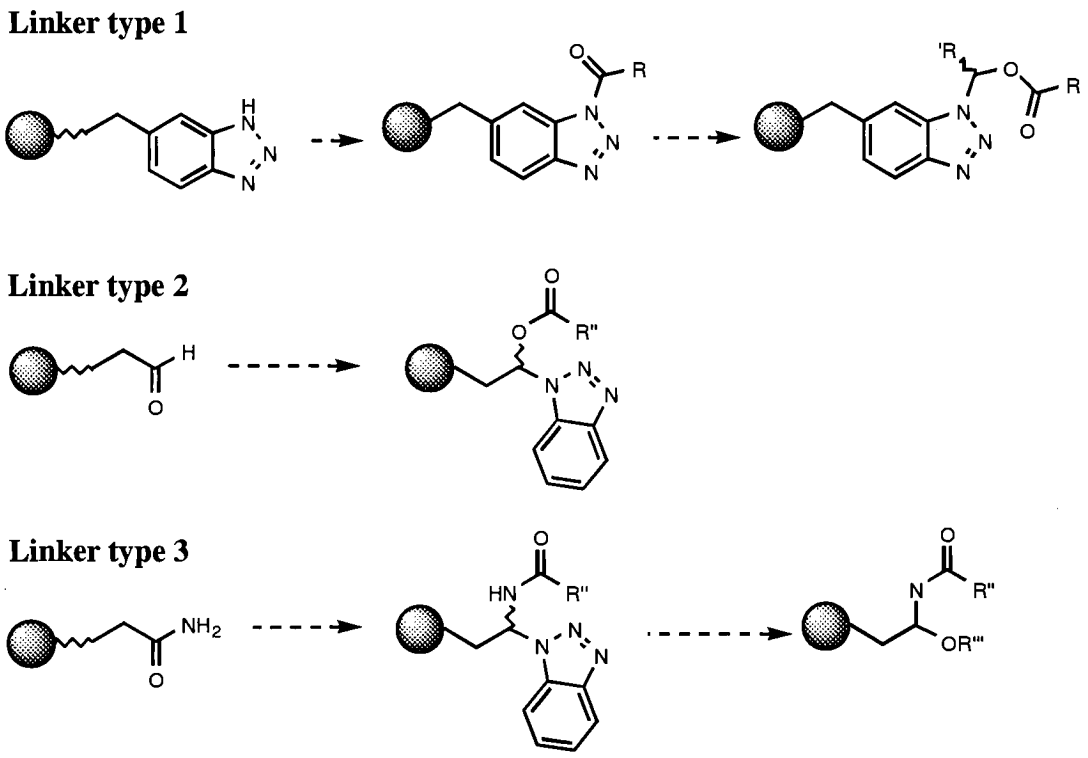
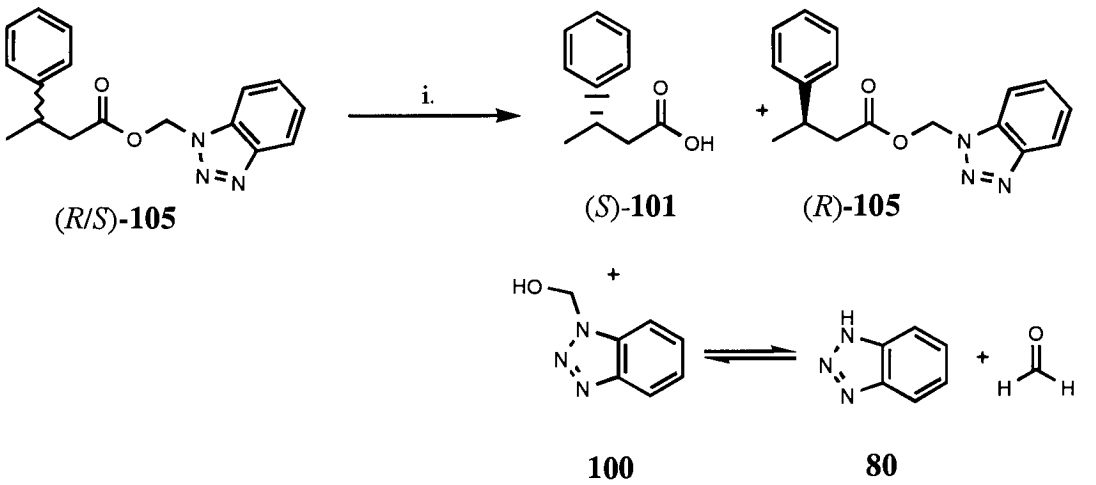


Figure 20: *Proposed synthesis of benzotriazole based enzyme cleavable linkers.*

2.2 Enzymatic studies on 1-(benzotriazol-1-yl) alkyl esters of chiral acids

(*R/S*)-3-phenylbutyric acid benzotriazol-1yl-methyl ester **105** was chosen as a substrate for more detailed lipase/esterase hydrolysis studies – **Scheme 53**.



(*R/S*)-**105** (*S*)-**101** (*R*)-**105**

100 **80**

Scheme 53: *Reagents and conditions* i. lipase/esterase, pH7 buffer, RT.

2.2.1 Screening of lipases/esterases.

As a starting point, enzymes from the Fluka Lipase Basic Kit and Fluka Esterase Basic kit were tested, to establish which enzymes exhibited activity towards (*R/S*)-**105** – **Scheme 53**. Small scale screening reactions (0.009mmol **105**) were performed with enzyme at room temperature in pH 7 buffer. The enzymatic reactions were carried out for 24 hours, quenched by addition of acetone, and analysed by reverse phase HPLC. The amount of benzotriazole released (area%) was calculated and displayed -**Table 7**.

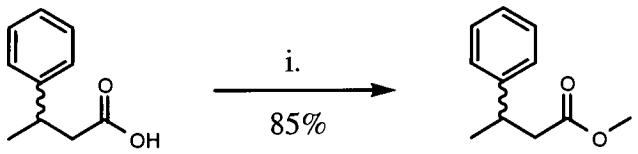
Lipase	Conversion	Esterase	Conversion
P. cepacia	100	*T. brockii*	++
Porcine Pancreatic	100	*S. cerevisiae*	+
A. niger	100	*C. lipolytica*	100
M. miehei	100	*M. miehei*	100
P. fluorescens	100	Hog Liver	100
R. niveus	++	Horse Liver	100
R. arrhizus	+	*B. thermoglucosidasius*	100
C. antarctica	++++	*B. stearthermophillus*	++++
C. cylindracea	+	*Bacillus sp.*	100
Control	-	Acetylcholine	++

Table 7: *Hydrolysis of (R/S)-**105*** + (0-25%), ++ (26-50%), +++ (51-75%), ++++ (76-99%).

No hydrolysis was detected in the control indicating that the ester was stable in pH 7 buffer. In addition, (*R/S*)-**105** was suspended in pH 7 buffer and analysed by HPLC every day for four days – no hydrolysis was detected after 4 days. Encouragingly, all 19 enzymes displayed activity towards (*R/S*)-**105** indicating that it was readily hydrolysed by both lipases and esterases.

2.2.2 Comparison of (*R/S*)-105 with (*R/S*) 3-phenylbutyric acid methyl ester

Attention was then turned to measuring the enantioselectivity of a selection of lipases and esterases towards (*R/S*)-**105** and comparison of the *E* values determined with those reported for the resolution of (*R/S*)-**104.** The methyl ester **104** was easily prepared by refluxing (*R/S*)-**101** in acidified methanol - **Scheme 54.**



(*R/S*)-**101** (*R/S*)-**104**

Scheme 54: *Reagents and conditions* i. methanol, cat. H_2SO_4, reflux

Using identical conditions (concentration of substrate, enzyme and pH of buffer) resolutions of (*R/S*)-**104** and (*R/S*)-**105** were run in tandem. The conversion, C (using previously determined relative response factors for both substrates) and the enantiomeric excess of the non-hydrolysed substrate were determined using reverse phase HPLC and chiral HPLC, respectively. Mean *E* values (from four separate determinations) were calculated using the method reported by Sih[156] and compared to those reported by Reeve[140] for the resolution of (*R/S*)-**104 - Table 8**.

Enzyme	*E*-(105)	*E*-(104)	Reported[140] *E* for 104
P. cepacia lipase	11.7	12.6	-
C. viscosum lipase	>133	136	>153
P. fluorescens lipase	29	-	>34
Hog liver esterase	1.1	1.1	2
Horse liver esterase	2.7	1.3	-
C. *lipolytica* esterase	2.3	2.2	-

Table 8: *Enantioselectivity of lipase and esterase catalysed hydrolysis of* (*R/S*) – **105** and (*R/S*)-**104**

The results obtained with these six enzymes are very encouraging indicating that the enantioselectivity displayed towards the benzotriazole ester **105** was similar to that

observed for the methyl ester **104**. For **104** and **105** the same enantiopreference was observed, with all enzymes hydrolyzing the (*S*)-ester more rapidly than the (*R*)-ester. These results are encouraging and indicate that these benzotriazole esters have potential as chromophoric substrates for screening enzymatic reactions. They could potentially be used in preference to the more regularly employed *p*-nitrophenyl esters which are known to undergo non-specific hydrolysis.[75] We then turned our attention to determining the initial rate of hydrolysis for (*R/S*)-**105** compared to (*R/S*)-**104**.

2.2.3 Comparison of initial rates of hydrolysis of 104 and 105

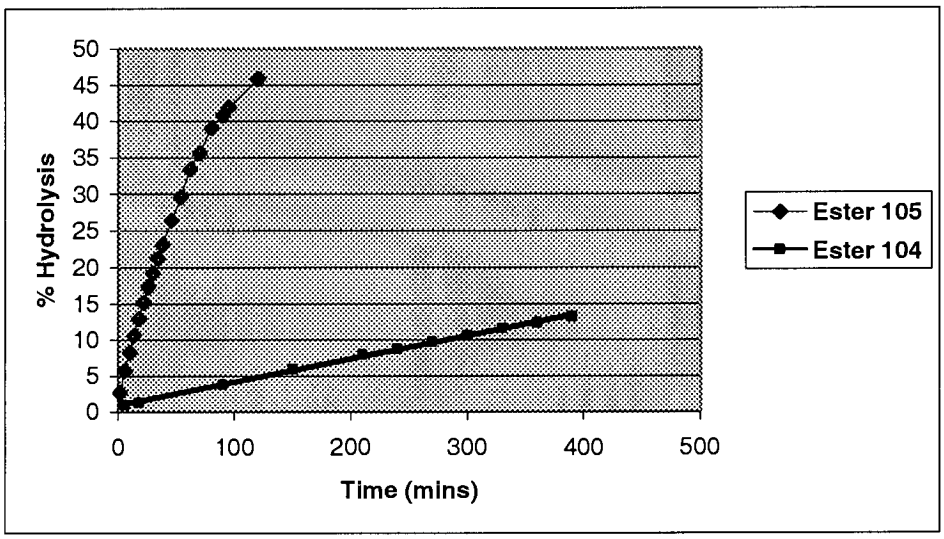
The initial rate of enzymatic hydrolysis of (*R/S*)-**104** and (*R/S*)-**105** was determined for the six enzymes used in the previous experiment – **Table 9**. The initial rate was measured for both esters, under identical conditions, using concentrations where both esters remained fully soluble – full experimental details are documented in **Chapter 5**.

Enzyme	**Initial rate for 104** (pmoles min^{-1})	**Initial rate for 105** (pmoles min^{-1})
P. cepacia lipase	1.6	62
C. viscosum lipase	0.5	150
P. fluorescens lipase	2.3	43
Hog liver esterase	6.0	214
Horse liver esterase	3.0	2
C. *lipolytica* esterase	1.6	0.5

Table 9: *Initial rates measured for the enzymatic hydrolysis of (R/S)-***104** *and (R/S)-***105**

The initial rates measured do not seem to follow any general trend. Four of the enzymes tested hydrolyse the benzotriazole ester **105** considerably faster than the methyl ester (Entries 1-4, Table 9). Horse liver esterase hydrolyses the esters at approximately equal rates and the esterase from *Candida lypolytica* hydrolyses the methyl ester more rapidly (Entries 5 and 6, respectively). Interestingly, despite this much higher hydrolysis

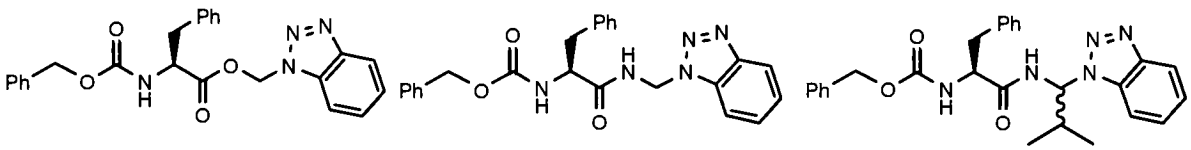
rate the lipase from *Chromobacterium viscosum* still exhibits a high enantioselectivity towards the (*S*)-isomer of the benzotriazole ester. A graph comparing the rate of hydrolysis of (*R/S*)-**104** with that of (*R/S*)-**105** using *Pseudomonas fluorescens* lipase is displayed - **Graph 1**. Due to a shortage of time no further investigations could be performed with the benzotriazole ester **105**.



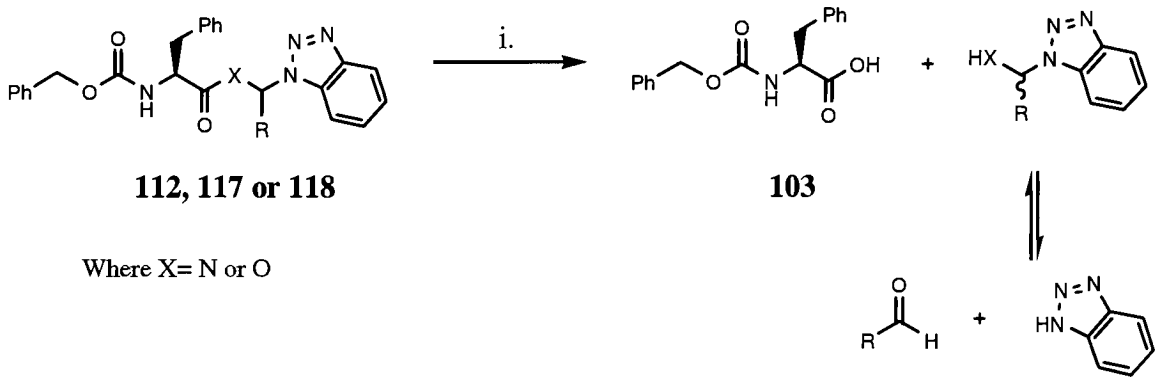
Graph 1: *Hydrolysis of* **104** *and* **105** *using Pseudomonas fluorescens lipase*

2.2.4 α–Chymotrypsin hydrolysis of ester 112 and amides 117 – 118

The benzotriazole containing compounds **112, 117** and **118** were proposed as substrates suitable for cleavage by the serine protease, α-chymotrypsin. **112, 117** and **118** were incubated with α-chymotrypsin at room temperature for 24 hours - **Scheme 55**.



112 **118** **117**



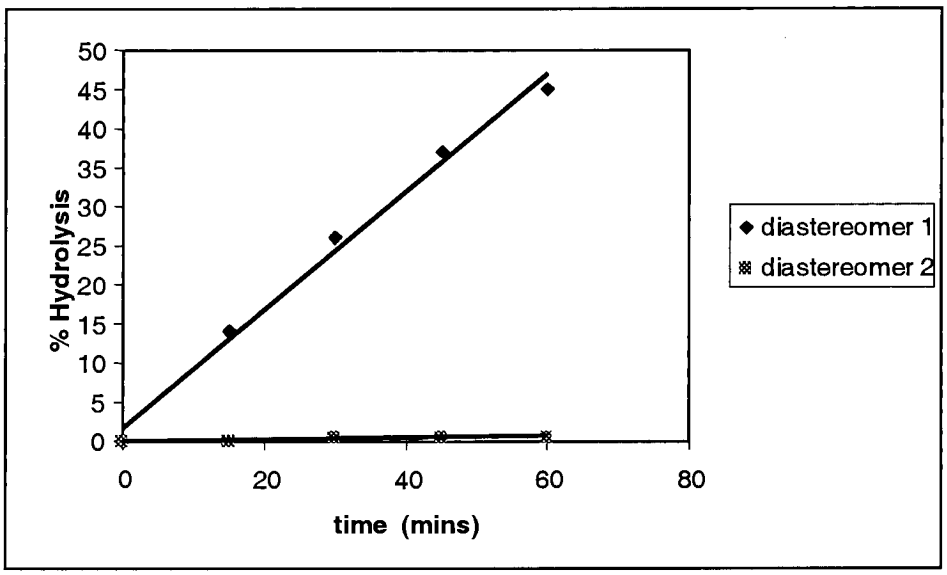
Scheme 55: *Reagents and conditions* i. α-chymotrypsin, pH 7.8 buffer, RT, 10%MeOH

The amount of hydrolysis of **112**, **117** and **118** was determined by quantification of the amount of Z-phenylalanine **103** released –**Table 10**. For all the amides a control was performed in tandem and in all controls no hydrolysis was detected indicating that the compounds were stable in the reaction buffer. As expected, ester **112** was hydrolysed much more readily than the equivalent amide **118** to release the acid and benzotriazole.

Entry	% Hydrolysis
112	100%*
118	68%*
117	57%
Controls	-

Table 10: *Hydrolysis by α-chymotrypsin** denotes area% result.

An interesting phenomenon, concerning the two diastereomers of **117,** was observed, notably one diastereomer was hydrolysed almost exclusively over the other diastereomer. This was examined further using reverse phase HPLC and relative rates of hydrolysis are displayed - **Graph 2**.



Graph 2: *α-chymotrypsin hydrolysis of the individual diastereomers of* **117**

The configuration of the faster reacting diastereomer of **117** was not identified. These results indicated that structures of this type, generated from an amide and an aliphatic aldehyde (other than formaldehyde) would be unsuitable as enzyme labile 'blocking' groups for SERRS dyes due to unequal reactivity of the diastereomers.

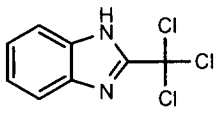
2.2.5 Summary

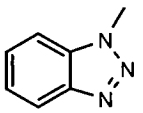
Enzymatic resolutions have been performed on (*R/S*)-**105** and (*R/S*)-**104**. *E* values determined for **105** were comparable with those determined for the methyl ester and with those previously reported by Reeve and co-workers.[140] Amides **112**, **117** and **118** were substrates for α-chymotrypsin although the individual diastereomers of **117** were hydrolysed at significantly different rates.

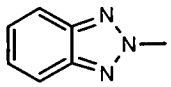
2.2.6 Future Work

One area of research which has attracted attention recently is the application of colour tests suitable for the detection of enzyme activity *e.g.* the use of 4-(*p*-nitro

benzyl) pyridine[78] to detect the products of epoxide hydrolases. The use of benzotriazole ester substrates *e.g.* **105** could offer similar opportunities. Konopski and Kielczewska reported[164] the use of 2-trichloromethylbenzimidazole as a sensitive dyeing reagent suitable for the detection of five-membered heteroaromatic compounds in TLC. 2-trichloromethylbenzimdazole was used to detect benzotriazole, through the generation of a yellow coloured compound, with detection limits of 0.3μg. Interestingly, this stain did not show any colour change with 1 or 2-methylbenzotriazole suggesting that the coloured compound generated occured via reaction at one of the triazole nitrogens. 2-Trichlorobenzimidazole could potentially be used to detect the benzotriazole released during the enzymatic hydrolysis of **105**. Chemical modification of 2-trichloromethylbenzimidazole may produce a stain that generates a more distinctive colour, with benzotriazole, than yellow.







2-trichloromethylbenzimidazole

Chapter 3 – Results and Discussion II

3.1 Application of developed synthetic methodology

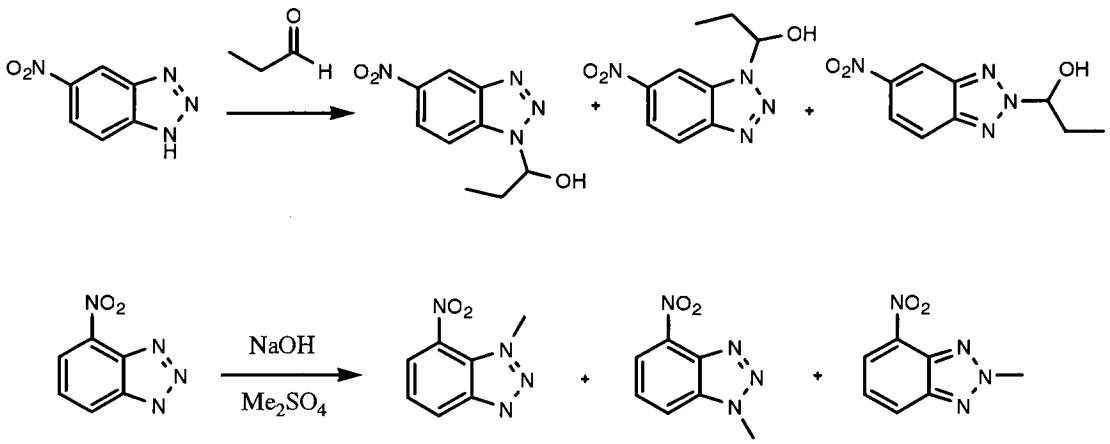
3.1.1 The synthesis of SERRS blocked dyes

The success of measuring enzymatic reactions using SERRS is dependant on the ability to quantify the concentration of one SERRS dye in the presence of another SERRS dye, without the need for a separation step. This requires that the dyes used have suitably distinct spectra, so that a unique peak from each spectrum can be used for quantification purposes. At the time of initiating this study very little data was available on the quantification of SERRS dyes in mixtures, and therefore several different dyes had to be synthesized. In light of this, a range of SERRS dyes will be generated in order to investigate their properties and the ease with which they could be converted into the 'blocked' dye - **Scheme 56**.

SERRS active dye 'Blocked' dye- **SERRS inactive**

Scheme 56: *Conversion of SERRS active dye to SERRS inactive dye*

The exact position of the azo linkage to the benzotriazole ring has not been defined because desymmetrisation of the benzotriazole rings leads to regioisomerism around the triazole ring. Several reports[129, 157, 158] have described the tautomerism of the hydrogen attached to the nitrogen in ring substituted benzotriazoles, two examples of which are shown in **Scheme 57.**



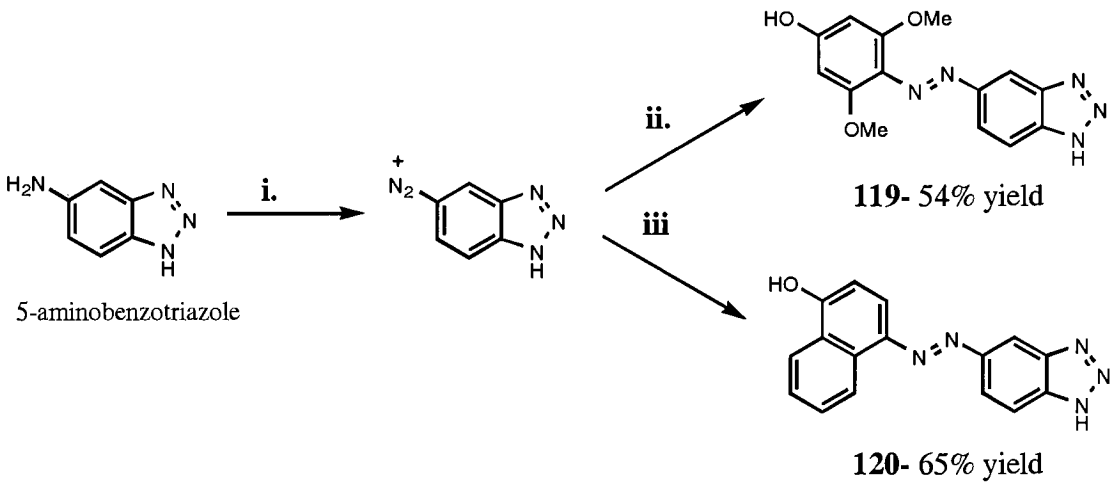
Scheme 57: *Regioisomerism in ring-substituted benzotriazoles*

At this point, it is important to note that it was decided to derivatise the benzotriazole ring after dye formation in order to introduce the 'valuable' chiral acid as close to the end of the synthetic pathway as possible. It was felt that if SERRS analysis of enzymatic reactions was to become a viable technique then the amount of enantiomerically pure acids required and the number of synthetic manipulations needed to obtain the ester derivatives should be minimised.

The dyes synthesised during this investigation can be divided easily into two distinct families; phenolic and *N'N'* dimethyl aromatic dyes. The syntheses of both are very similar but they will be discussed independently for clarity.

3.1.2 Synthesis of phenolic monoazo-benzotriazole dyes

Starting from commercially available 5-aminobenzotriazole, dyes **119** and **120** where synthesized by diazotisation with $NaNO_2$ in conc. HCl followed by coupling with the appropriate activated aromatic phenol in sodium hydroxide solution (1M) containing sodium acetate - **Scheme 58.**



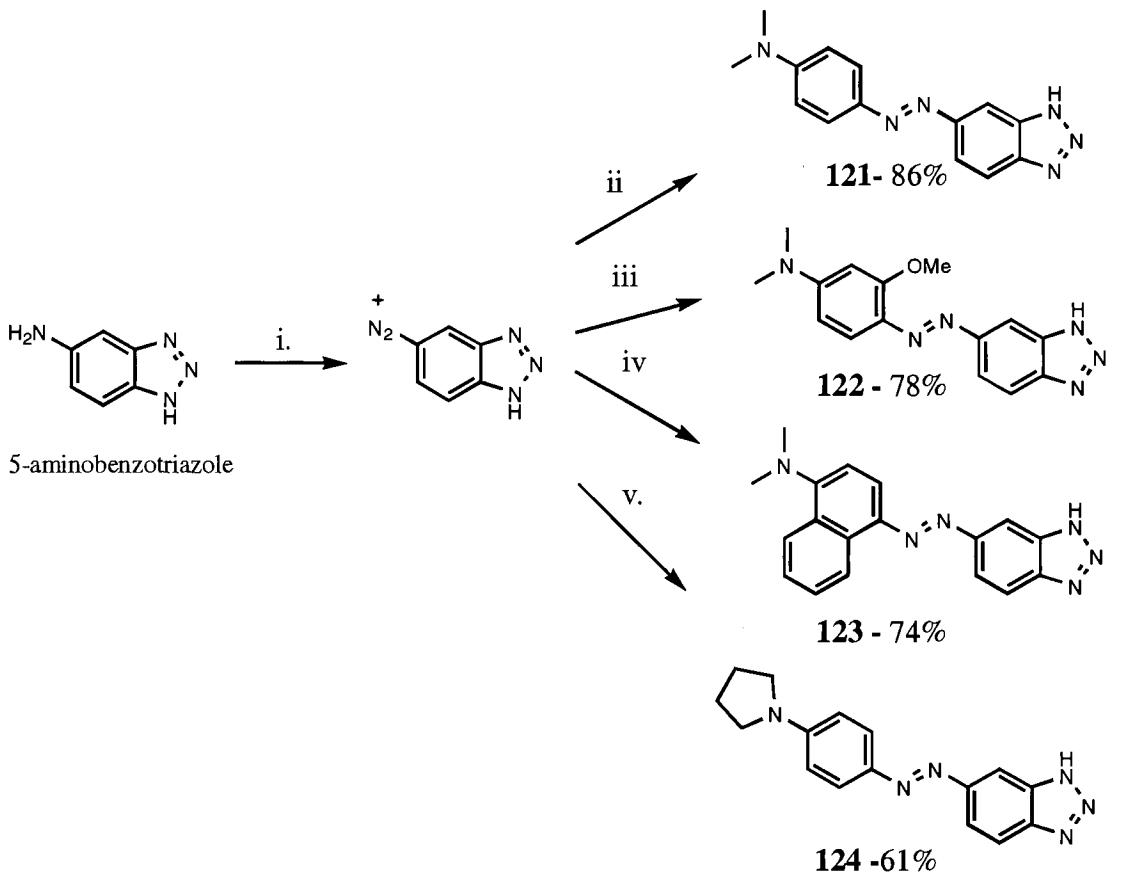
Scheme 58: *Reagents and conditions* i. $NaNO_2$, HCl (36%), ii. 2,4-dimethoxyphenol, NaOH (1M), sodium acetate, iii. α–naphthol, NaOH (1M), sodium acetate.

The yields of **119** and **120** were moderate, at 54 and 65%, respectively. The low yields were attributed primarily due to problems experienced during purification. Column chromatography on silica gel was problematic due to the very limited solubility of the dyes in most suitable organic solvents and the poor elution characteristics of the dyes when pre-adsorbed on to silica. The dyes were obtained as highly coloured amorphous powders after extensive trituration in methanol, DCM and diethyl ether in accordance with methods reported by Graham *et al.*[121] The dyes have been designated as the 6'-isomer although no crystal structure could be determined due to poor crystal formation. This will be discussed in more detail in **Section 3.1.3.**

3.1.3 Syntheses of *N'N*-dimethyl aromatic monoazo-benzotriazole dyes

Following the successful synthesis of dyes **119** and **120**, the aromatic compound coupled to 5-aminobenzotriazole was modified; replacing the phenol with an *N'N'*-dimethyl moiety **121-123** or pyrollidine ring **124**. Three different *N'N'*-dimethyl aromatic compounds were chosen, *N'N'*-dimethylaniline, 3-dimethylaminoanisole and *N'N'* dimethylnapthylamine to generate dyes **121**, **122** and **123** respectively. The

aromatic compounds were chosen specifically to produce dyes that would give very different SERRS spectra from each other and therefore allow discrimination between them. The introduction of the methoxy group or incorporation of a fused aromatic ring should shift the absorbance maximum of dyes **122** and **123** to a longer wavelength with respect to dye **121**. This shift was reported[121] for dyes generated from phenylamine analogues. The longer absorbance maxima dyes generally display enhanced sensitivity due to an increase in resonance generated by the excitation laser (514nm). Dye **124** was synthesised to investigate if any non-specific interactions occur between the *N'N'*-dimethyl moiety and the negatively charged surface of the colloid – See **Chapter 4** for further discussion in this area. The dye synthesis is displayed - **Scheme 59.**



Scheme 59. *Reagents and conditions* i. $NaNO_2$, conc. HCl (36%), ii, iii, iv,v appropriate aromatic compound, NaOAc(1M).

Dyes **121-124** were synthesized in a similar manner to **119** and **120,** except the coupling reaction is carried out below pH 7. In general, yields were higher than the phenol dyes, ranging from 61% for **124** to 86% for **121**. **121-124** were all isolated as highly coloured amorphous powders. Purification was achieved by trituration in DCM, methanol and diethyl ether but column chromatography on silica was possible because the dyes displayed much better elution properties than the phenolic dyes.

Recrystallisation of the dyes was attempted but only **122** gave crystals, from an acetone/hexane mixture, which could be used for x-ray crystal diffraction studies. Recrystallisation was attempted from a variety of other solvents but the dyes displayed very poor crystal formation, a phenomenon which was reported by Graham *et al.*.[121] The crystal structure of dye **122** was obtained (see **Appendix 7.1** for full details) and is represented in **Figure 21**. The structure clearly indicates that this dye exists as the 6-isomer. This suggests that the 6-isomer is lower in energy than the 5-isomer once functionalised. This structure is consistent with those obtained for similar monazo-benzotriazole dyes and with that reported[160] for 6-(6'-azobenzotriazolyl)-7-methoxy-2,2,4-trimethyl-1,2-dihydroquinoline **125** which is currently the only published structure. The azo bond was defined as existing with *trans* geometry.

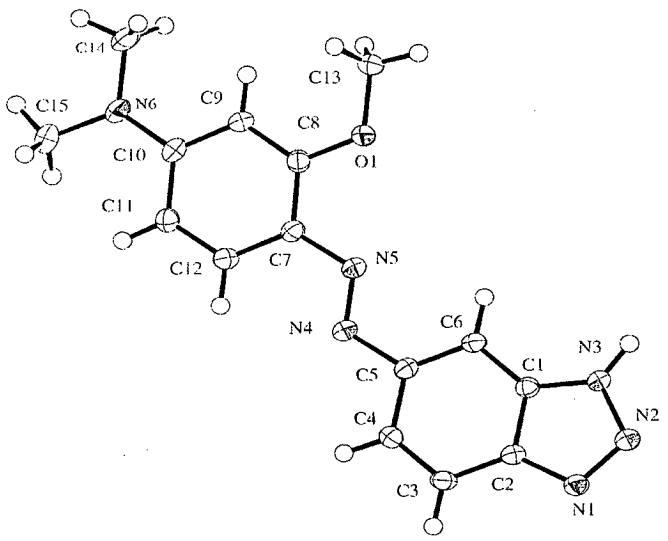
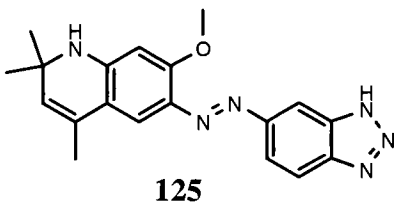


Figure 21: *X-ray crystal structure of dye* **122**

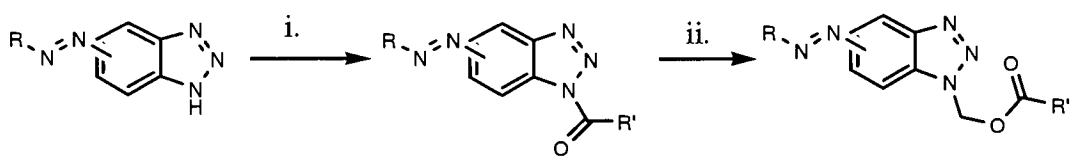


125

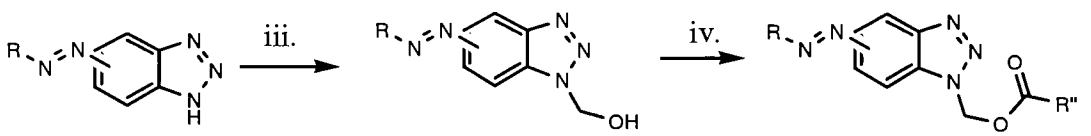
4.3 Proposed routes to 'blocked' monoazo-benzotriazole dyes

With six different dyes in hand **119-124** investigations began into their conversion into the 'blocked' derivatives. From preliminary studies on benzotriazole (Section 2.1) two routes existed for the preparation of the esters - **Scheme 60**.

ROUTE A



ROUTE B



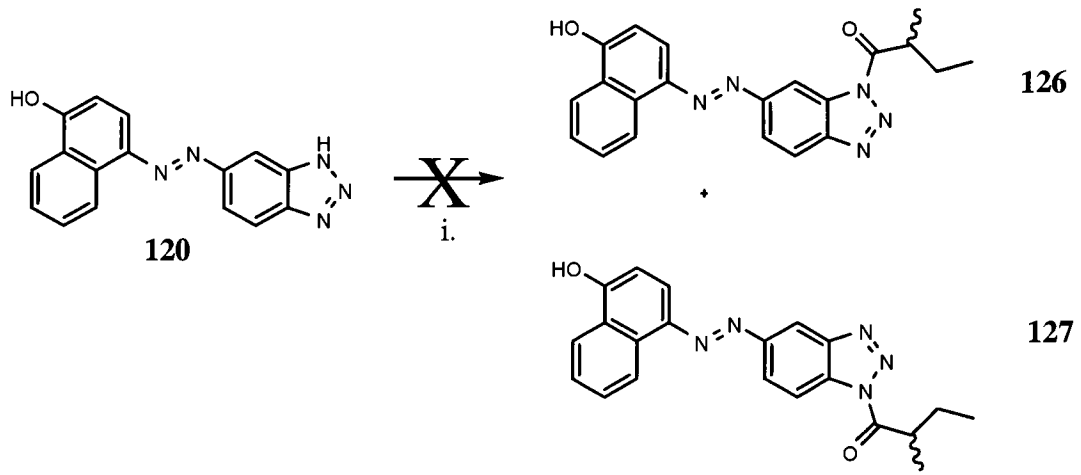
Scheme 60: *Proposed routes to blocked dyes* **A** *i.* R'COCl, Et_3N ii. HCHO, CH_3CN, K_2CO_3. **B** iii. H^+, HCHO, solvent, iv. coupling reagent, R''COOH

Both routes **A** and **B** consisted of two synthetic steps and would allow incorporation of a range of chiral acids either as acid chlorides or as the parent acid. It was decided to investigate route **A** with the both the phenolic dye **120** and the *N'N'*-dimethyl aniline dye **121**. Consideration had been made of the nucleophilicity of the phenolic OH but Graham *et. al.*[121, 130] had reported that the nucleophilicity of the amine

moiety of a similar dye, generated from aniline, was dramatically reduced due to electron donation into the ring system.

3.1.4 Acylation of monoazo-benzotriazole dyes

Acylation of dye **120** was attempted with (*R/S*) 2-methylbutryl chloride **109** in an attempt to form the *N*-acyl derivatives **126** and **127** - **Scheme 61**.



Scheme 61 *Reagents and conditions* i. (*R/S*) 2-methylbutyryl chloride, Et_3N, anhydrous DCM, 0°C

The above reaction generated a very complex mixture of compounds, which were inseparable by column chromatography (due to the poor running properties of the dye). Preparative HPLC allowed the isolation of 4 main peaks from the reaction mixture, 2 of which gave interpretable NMR data and were identified as **128a+128b** and **129 – Figure 22. 128 (a+b)** were isolated as a mixture of both regioisomers, eluting as a single peak.

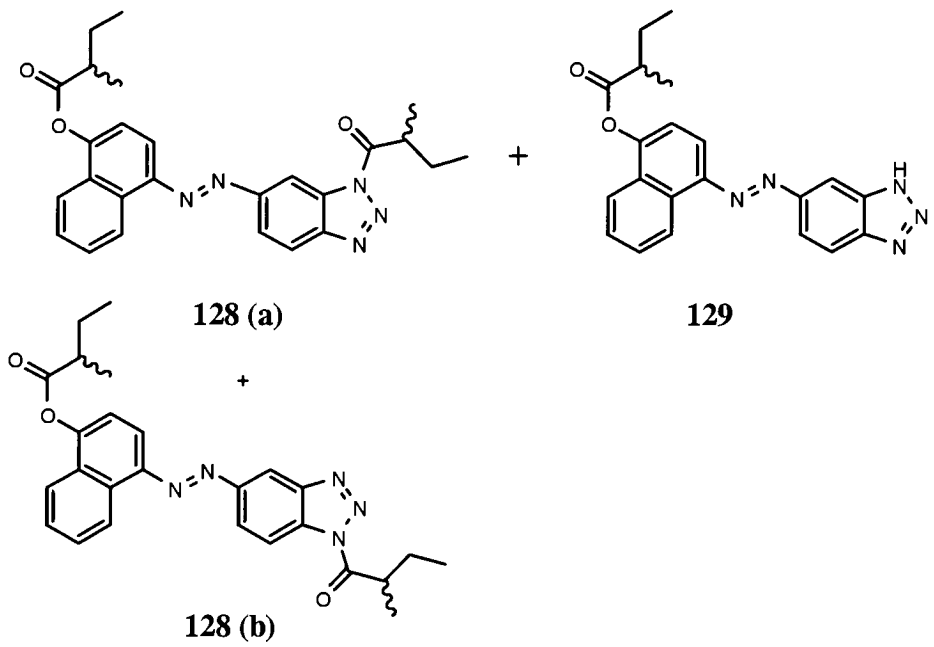
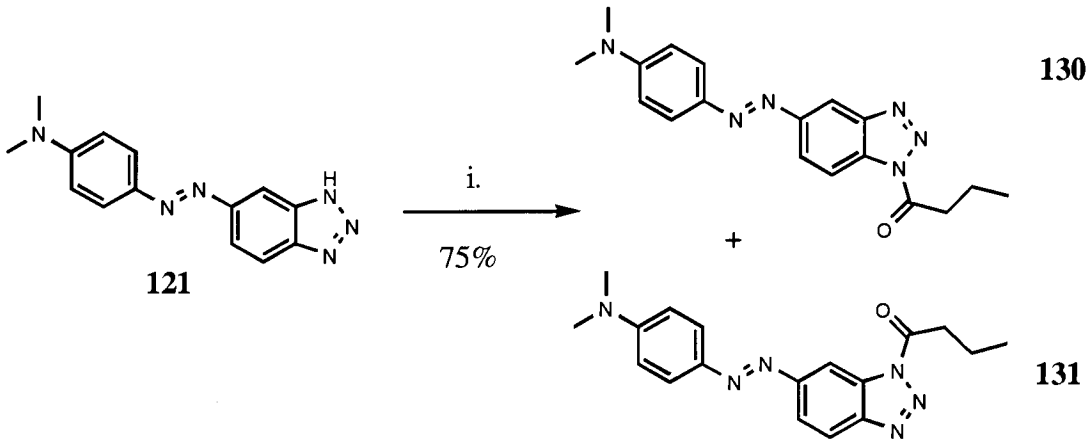


Figure 22: *Compounds isolated from acylation of dye* **120**.

The 1H NMR's of the other two compounds did not indicate that they contained acylated benzotriazole rings, *i.e.* neither contained a signal at approximately 4 p.p.m which was present in the corresponding benzotriazole compound, (*R/S*)-**110**. The acylation reaction was repeated and the crude mixture treated with formaldehyde in CH_3CN (step ii. in **Route A, Scheme 60**) but no 'ester' compound was detected by mass spectroscopy. In a similar manner, **119** was acylated with acid chloride **109** and again very complex mixtures were generated.

In sharp contrast to this, acylation of dye **121** with butyryl chloride generated a mixture of the *N*-acyl derivatives **130** and **131** - Scheme **62**. The yield of **130** and **131** combined was 75% and the ratio of regioisomers was approximately 1:1, as calculated from comparison of the ^1H NMR signals from the isolated benzotriazole ring proton.



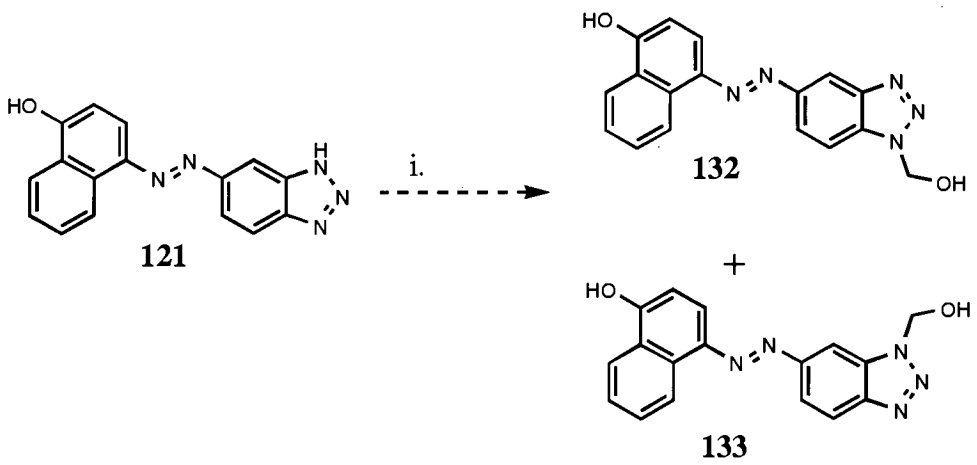
Scheme 62: *Reagents and conditions*; i. butyryl chloride, DCM, Et_3N, O°C

The crude reaction mixture was relatively clean, compared with the complex mixture generated for the acylation of dye **120**, consisting of only **130** and **131** and unreacted **121**. Separation of the unreacted dye was easily accomplished by column chromatography but isolation of each regioisomer could not be achieved using this technique. However isolation of the regioisomers was possible by reverse phase preparative HPLC on a Biotage FLEX system. Separation was achieved on a small scale using injections of a mixture of **130** and **131** at a maximum concentration of 5mg/ml. The separation was however, very time consuming due to gradual loss of baseline resolution during the purification process and slight on-column degradation. The acyl derivatives **130** and **131** were shown to be water sensitive, although any degradation products could be removed easily by further silica gel chromatography. Following the success of this reaction **121** was acylated with (*R*) and (*S*)-3-phenylbutyryl chloride – this is discussed in **Section 3.2.1**.

3.1.5 Preparation of 1-hydroxymethyl derivatives of phenolic SERRS dyes.

Despite the synthetic problems encountered using the phenolic dyes it was decided to continue with their use and attempt to synthesize the ester derivatives via the hydroxymethyl derivative – *i.e* Route B - **Scheme 59**. The analogous reaction with benzotriazole is well documented[139] and proceeded easily with precipitation of 1-

hydroxymethylbenzotriazole **100** in almost quantitative yield. It was envisaged that this could easily be applied to the monoazo-benzotriazole dyes therefore dye **121** was reacted with formaldehyde in an attempt to form the hydroxymethyl derivatives **132** and **133 – Scheme 63.**

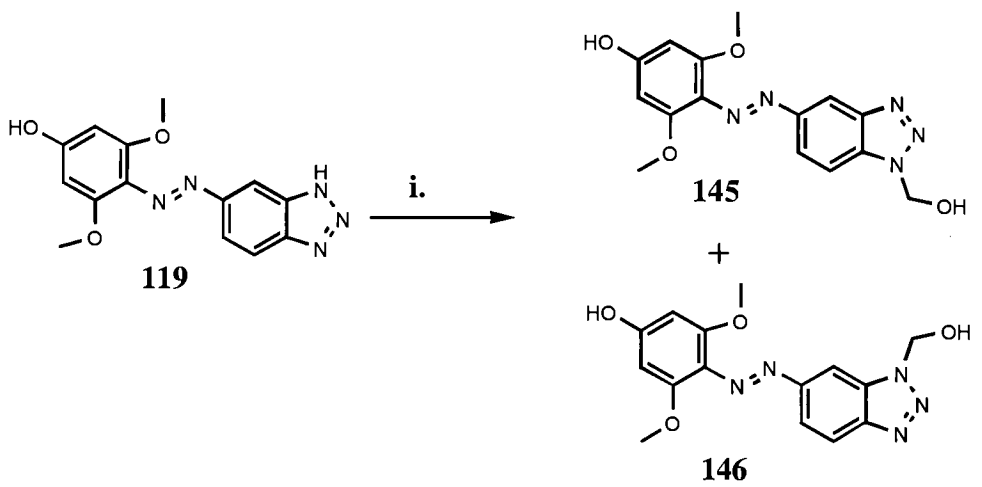


Scheme 63: *Reagents and conditions* i. acetone, acetic acid, H_2O, formaldehyde

121 was found to be insoluble in aqueous acetic acid, so acetone was added to aid solubility and the reactions were heated. A highly coloured precipitate was recovered on removal of the acetone, isolated and analysed by NMR. The ^1H NMR of **132/133** gave a doublet at 6.20 p.p.m (J= 11.2Hz) which was assigned to the $C\underline{H}_2OH$ doublet signal although the integral corresponded to only 1.4Hz. This compared favourably with the $C\underline{H}_2OH$ signal previously seen in **100** which resonated at 6.0 p.p.m (*J*= 7.3Hz). Further evidence was noted in the ^{13}C NMR with **132/133** displaying two $\underline{C}H_2OH$ signals at 70.5 and 70.6 p.p.m, which is practically identical to that obtained for **100** ($\underline{C}H_2OH$ at 70.4 p.p.m). Unfortunately, the other regions of the ^1H NMR spectra were broad and complicated. This was attributed to the precipitate containing both **132/133** and the unreacted dye **121**. Column chromatography on silica was problematic because **132/133** eluted at the same time as the unreacted dye and did not chromatograph well. Column chromatography was attempted, by Kirk Malone as part of an Honours project, and resulted in the isolation of the parent dye only. HPLC analysis of **132/133** gave two

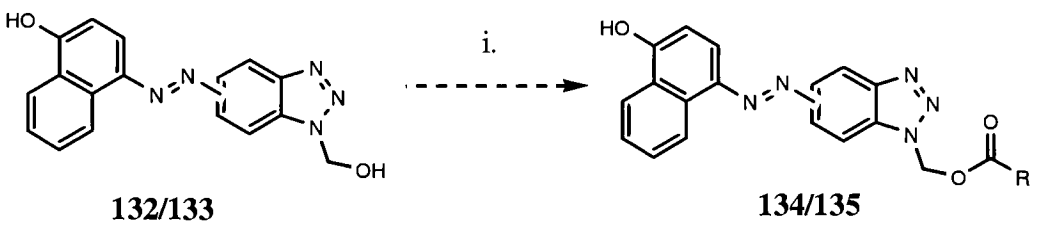
peaks, one of which had a similar retention time to the azo dye. The other peak disappeared after two hours on standing in CH_3CN suggesting that the other product was unstable in solution. In an attempt to obtain pure **132/133** the reaction conditions were varied including changing the acetic acid to trifluoroacetic acid, acetone to other organic solvents and performing the reaction at room temperature. In all cases no pure products could be isolated.

A similar situation was observed for the synthesis of hydroxymethyl derivative **145/146** from dye **119 - Scheme 64**. Again pure product could not be isolated but NMR analysis of the crude products displayed similar resonances to those which had been observed for **132/133** *i.e.* the CH_2OH group resonated at 70.4 + 70.5 p.p.m in ^{13}C NMR and 6.17 p.p.m in ^{1}H NMR.



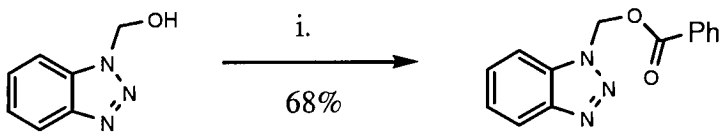
Scheme 64: *Reagents and conditions* i. acetone, acetic acid, H_2O, formaldehyde.

Despite the failure to isolate a pure sample of **132/133** a number of attempts were made to convert the suspected 1-hydroxymethyl derivatives into the esters **134/135** according to **Scheme 65**.



Scheme 65: *Regents and conditions* – i. See **Table 11**.

Katritzky *et al.*[161] reported successfully acylating 1-hydroxymethylbenzotriazole using benzoyl chloride in pyridine with 10mol% DMAP to give the ester **136** - **Scheme 66.**



100 **136**

Scheme 66: *Reagents and conditions* i. PhCOCl, DMAP, pyridine.

Using the above conditions, and a range of other conditions, which had previously been shown to be successful with 1-hydroxymethylbenzotriazole (**Section 2.1.7**) the conversion of the impure mixture of **132/133** to esters **134/135** was attempted **–Table 11**. HPLC and mass spectrometry was used to follow the progress of each reaction. It was hypothesized that the esters **134/135** would be less polar than **132/133** and as such would elute later using reverse phase HPLC. In all reactions where later eluting products were observed, attempts were made to isolate and identify these products.

Entry	R	Conditions	Comments
1	$CH_3CH_2CH_2COCl$	(cat.)DMAP, DMF	2 products by HPLC
2	$CH_3CH_2CH_2COCl$	(cat.) DMAP, py, DMF	2 products by HPLC
3	$CH_3CH_2CH_2COCl$	Et_3N, DCM	2 products by HPLC
4	$CH_3CH_2CH_2COCl$	DIPEA, DMF	2 products by HPLC
5	$(CH_3CH_2CH_2CO)_2$	(cat.)DMAP, py, THF	2 products by HPLC
6	(+/-) $CH_3PhCHCH_2COOH$	PPh_3, DEAD, DMF	No reaction by HPLC/MS
7	(+/-) $CH_3PhCHCH_2COOH$	EDCI, DMAP, DMF	No reaction by HPLC/MS
8	(+/-) $CH_3PhCHCH_2COOH$	DCC, DMAP, DMF	No reaction by HPLC/MS

Table 11: *All reactions carried under anhydrous conditions* ('py' denotes pyridine)

Using the conditions in entry **1** and **2 (Table 11)** two less polar products were detected but mass spectroscopy did not indicate the formation of the ester. The amount of products formed was greater for entry 1 than 2 (55% compared to 15.5%). The products formed were found to be very unstable in the reaction mixture and degraded over time. Reaction **1** was repeated, quenched after 1 hour and the 2 less polar products isolated by preparative scale HPLC- full experimental details are given in **Chapter 5**. Following isolation, the 2 products were found to be contaminated with the parent dye **120** indicating that that they were degrading. ^{1}H NMR of the isolated compounds were very poor and neither displayed the $C\underline{H_2}OC{=}O$ signal expected (between 6.4 and 6.6 p.p.m). This process was repeated with the removal of TFA from the mobile phase but the isolated products were still unstable during isolation.

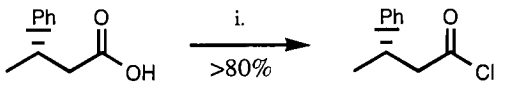
For reactions **3-5** a similar process was performed with the components again breaking down on isolation. It was postulated that at least one of the isolated products was probably the *N*-acyl derivative. To test this hypothesis, the isolated products from reaction **4** were treated with formaldehyde and K_2CO_3 in CH_3CN in an attempt to form the desired ester – unfortunately this led to breakdown of the product to the parent dye. In a second experiment, the isolated products were treated with *Candida antarctica* lipase in pH7 buffer to determine if either was the ester. Again this resulted in the breakdown of the products to the parent dye, however this was mirrored in the control indicating it was the action of the medium and not the enzyme. Due to a shortage of time, and failure of reactions with **132/133** the conversion of **145/146** to the ester was not attempted.

At this point, it was decided to abandon the use of the phenol dyes **119-120**, and concentrate on dyes **121-124.** In light of the success achieved in acylating **121** with butyryl chloride - **Scheme 62** it was decided that investigations would continue on the acylation of these dyes with (*S*) and (*R*)- 3-phenylbutyryl chloride **106**.

3.2 Generation of *pseudo*-enantiomeric blocked SERRS dyes

3.2.1 Acylation of dyes 121-124 with (*S*) and (*R*)-3-Phenylbutyryl chloride.

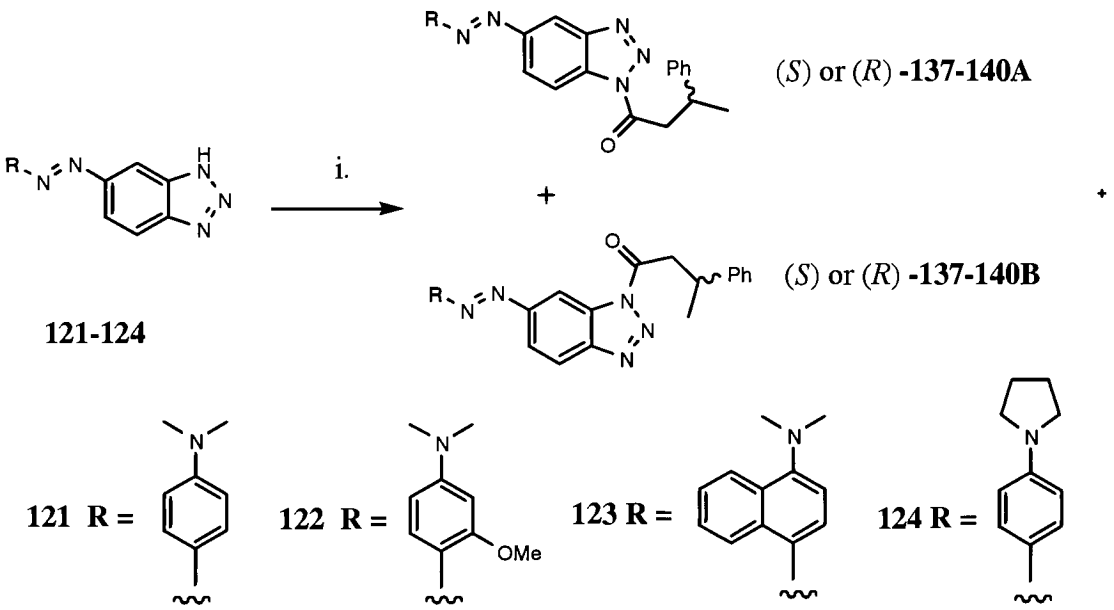
Commercially available (*S*)-and (*R*)-3-phenylbutyric acid **101** were converted to the corresponding acid chlorides (*S*) and (*R*) **106** in excellent yield using thionyl chloride or oxalyl chloride – **Scheme 67**.



(*S*) or (*R*) -**101** (*S*) or (*R*) -**106**

Scheme 67: *Reagents and conditions* i. $SOCl_2$ / $COCl_2$, reflux, cat. DMF.

The enantiopure acid chlorides **106** were then used to acylate dyes **121-124** in good to excellent yield to give the *N*-acyl dervatives (*S*) and (*R*)– **137** to **140** (**A** and **B**) - **Scheme 68.** (Note - Dye **124** was reacted with (*S*)-**106** only.) The yields and ratios of regioisomers formed in the reactions are displayed - **Table 12**.



Scheme 68: *Reagents and conditions i.* (*R*) or (*S*) **106**, Et_3N, DCM, 0°C, 1 hour.

Entry	Dye	Acid Chloride	Yield (A+B)	Ratio of regioisomers A	B	Separation of A + B
(*S*)-**137**	**121**	(*S*)-**106**	54%	48	52	√
(*R*)-**137**	**121**	(*R*)-**106**	68%	48	52	-
(*S*)-**138**	**122**	(*S*)-**106**	84%	44	56	-
(*R*)-**138**	**122**	(*R*)-**106**	83%	45	55	-
(*S*)-**139**	**123**	(*S*)-**106**	57%	50	50	-
(*R*)-**139**	**123**	(*R*)-**106**	85%	50	50	-
(*S*)-**140**	**124**	(*S*)-**106**	49%	50	50	-

Table 12 – *Yields of N-acyl derivatives of dyes 121-124*

All the *N*-acyl derivatives were formed as approximately equimolar mixtures of the 5' (**A**) and 6' (**B**) regisomers which were inseparable by silica gel chromatography. Preparative HPLC separation of (*S*)-**137A** + (*S*)-**137B** using a Biotage Flex system was successful - **Figure 23** although this process was time-consuming due to poor resolution of the regioisomers. The shaded area shows the fractions collected from the analyses – any fractions containing both peaks were pooled and re-separated.

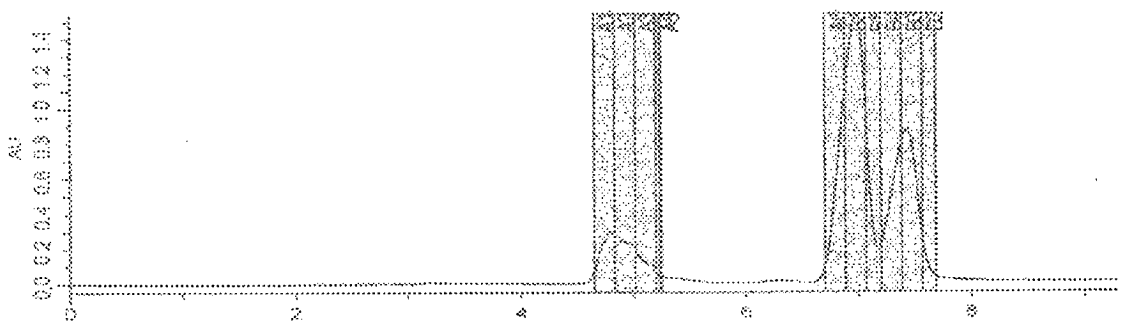


Figure 23: *Preparative HPLC separation of* (*S*)-**137A** *and* (*S*)-**137B**

NMR data from isolated samples of (*S*)-**137A** and (*S*)-**137B** was used to confirm that the other *N*-acyl derivatives were mixtures of the 5' and 6' isomer. Separation of (*S*)-**138A** + (*S*)-**138B** could not be achieved despite extensive method development.

Separation of the other acyl derivatives (**139A+B** and **140A+B**) was not attempted due to 'scrambling' of the regioisomerism during the next step as discussed in **Section 3.2.2.**

(*S*)-**137B** was confirmed as the 6' isomer by crystallisation of the isolated sample from CH_3CN and x-ray crystal structure determination - **Figure 24** and full details in **Appendix 7.2**. The crystals of (*S*)-**137B** were of insufficient size to provide an accurate hand determination. As can be seen from **Figure 24** the azo bond adopts *trans* geometry. (*S*)-**137A** produced crystals, which were very small and did not diffract sufficiently to provide a crystal structure. (*S*)-**137A** was assigned as the 5' isomer because acylation of the middle nitrogen of benzotriazole has not been reported in the literature.

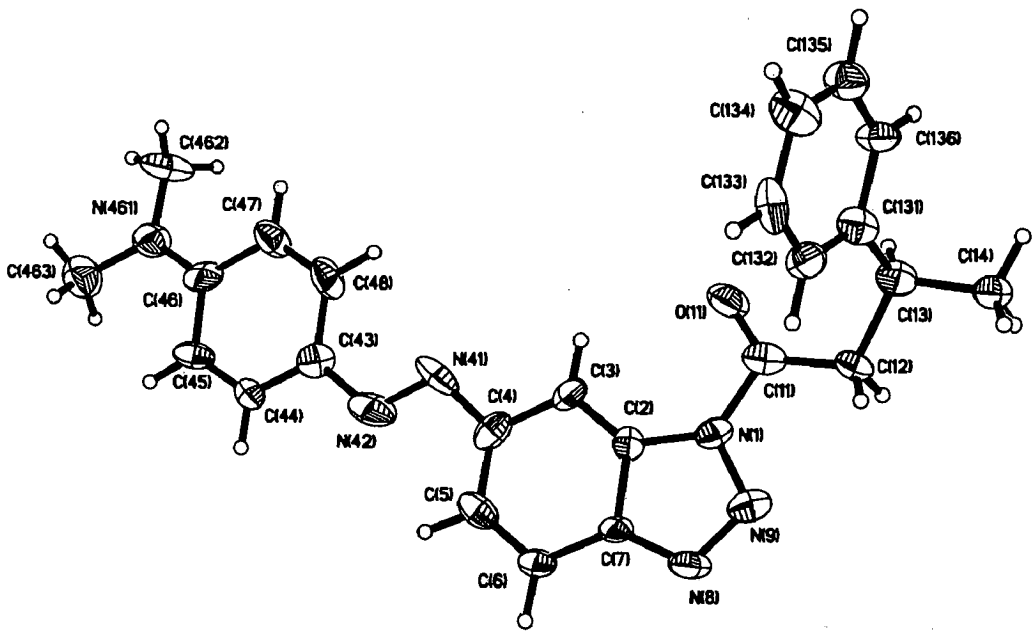
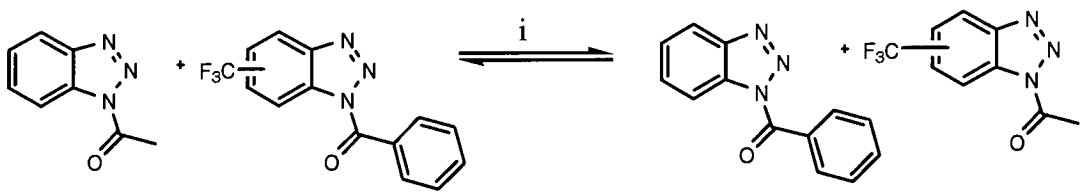


Figure 24: *X-ray crystal structure of (S)-137B*

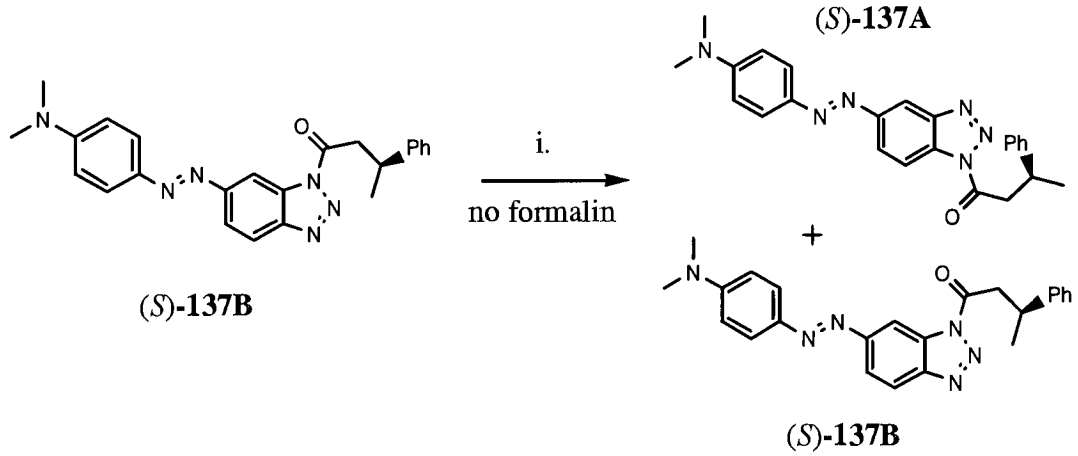
3.2.2 Conversion of *N*-acyl SERRS dyes into esters

The mechanism, by which an *N*-acyl benzotriazole (*e.g.* (*S*)-**137B**) is converted to the ester, by reaction with formaldehyde, is unknown. It is known[162] however, that *N*-acylated benzotriazoles undergo intermolecular thermal isomerisation and can act as weak acylating agents – **Scheme 69**.



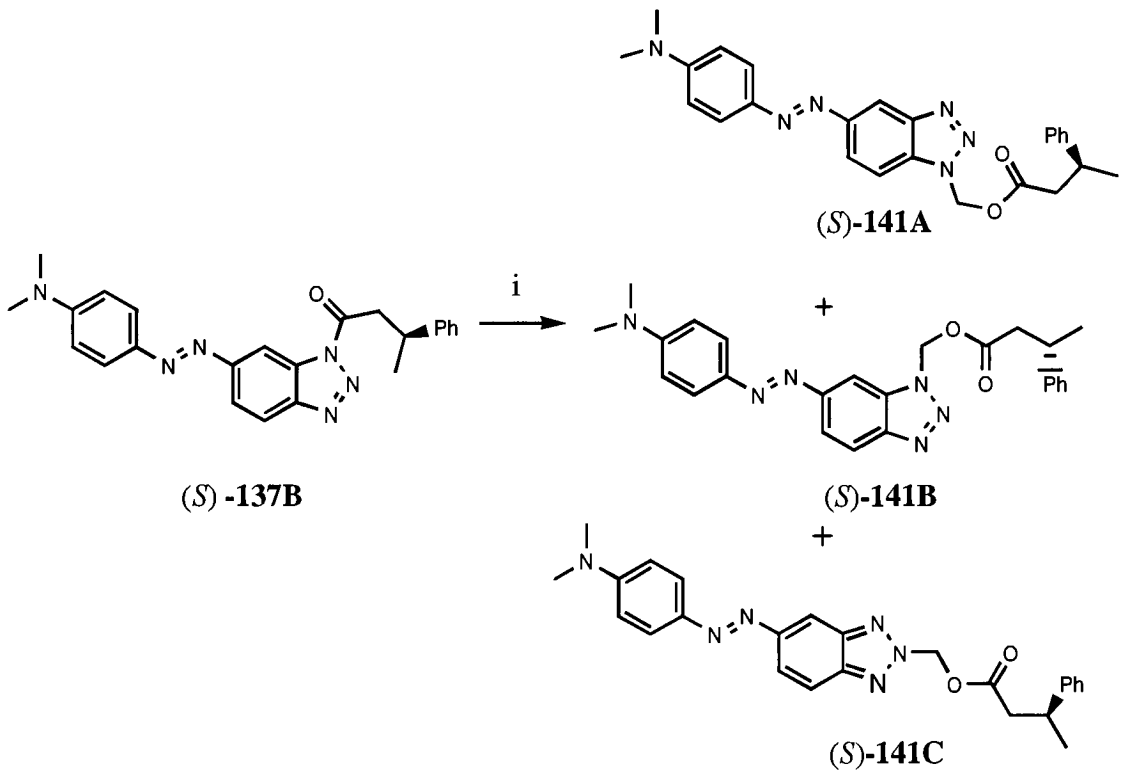
Scheme 69: *Reaction conditions* i. *O*-xylene under N_2

With this in mind, the single regioisomer (*S*)-**137B** was suspended overnight in CH_3CN with 15 mol% K_2CO_3 in either the presence or absence of formaldehyde. The outcome of each reaction is displayed in Schemes **70** and **71** for clarity.



Scheme 70: *Reagents and conditions*, i. K_2CO_3, CH_3CN, RT

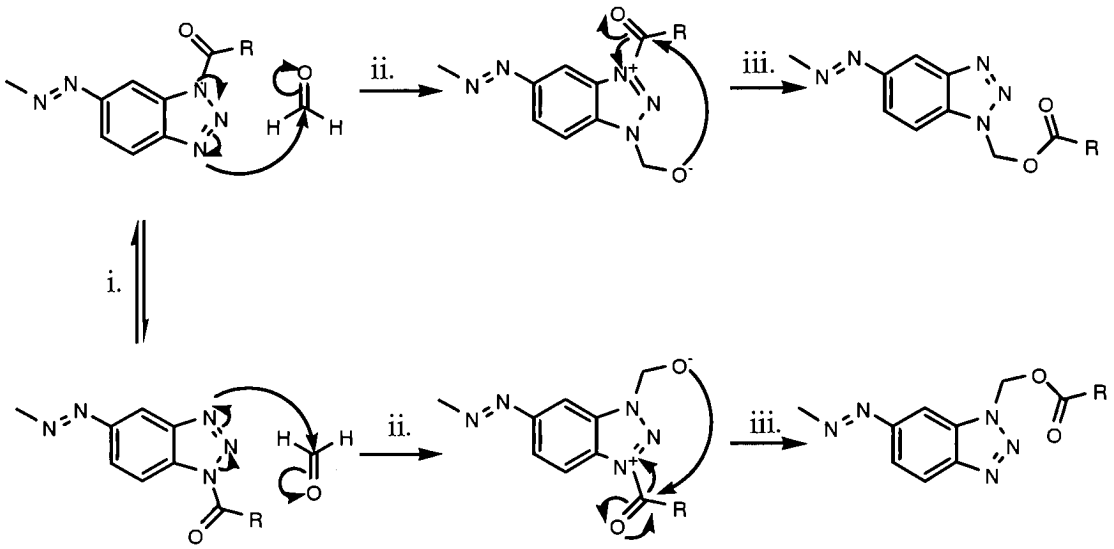
The results shown in **Scheme 70** indicated that the *N*-acyl benzotriazole (*S*)-**137B** undergoes an acyl transfer rearrangement under the reaction conditions used in the formation of the ester. After 16 hours suspension in CH_3CN with K_2CO_3 the ratio of (*S*)-**137B** to (*S*)-**137A** was 43:57 as determined by integration of the isolated ring proton in both species.



Scheme 71: *Reagents and conditions* i. K_2CO_3, CH_3CN, formaldehyde

The results displayed in **Scheme 71** show that all 3 possible regioisomers of the ester are formed – (*S*)-**141A**, **141B** and **141C**. Esters (*S*)-**141A** and (*S*)-**141B** were isolated by preparative HPLC and the ratio was determined as 39:61, respectively. (*S*)-**141C** was not isolated due to the small scale of the reaction (0.03mmols) although TLC of the crude reaction mixture confirmed its presence. These results do not provide a definitive indication of the mechanism of the reaction (the intermolecular acyl transfer, Step **i,** makes definitive mechanism determination very difficult) but the following mechanism is proposed - **Scheme 72**. Step **iii**. could be either intra-molecular or inter-molecular making determination of the mechanism more difficult. Step **iii.** would most likely occur intermolecularly due to the planer nature of the benzotriazole ring. Measurement of the kinetics of the reaction may shed more light on the mechanism but this was outwith the scope of this work. Note: IR analysis of the N-acyl benzotriazole

(*S*)-**137B** shows that the carbonyl stretch is at 1740cm^{-1} indicating that it is more ketone-like in character than a typical amide.

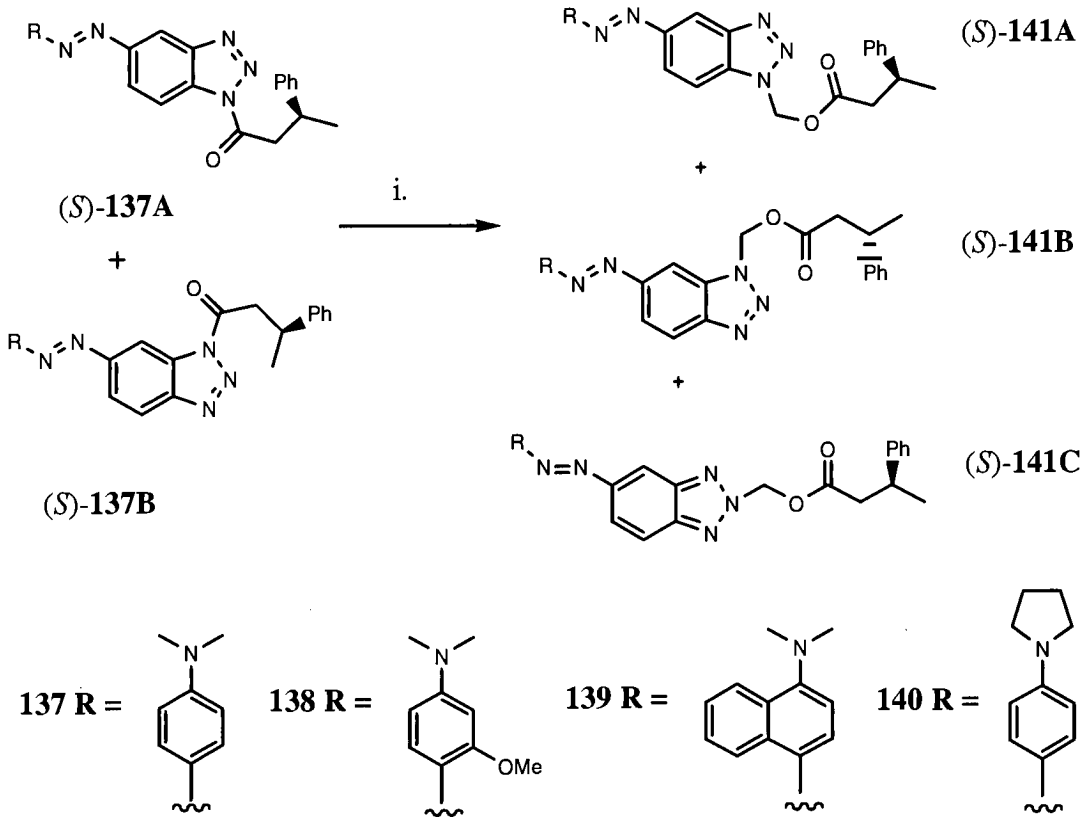


Scheme 72: *Proposed ester formation mechanism*

Due to the observed 'scrambling' of regioisomerism, a mixture of both *N*-acyl benzotriazoles was converted to the esters – **Scheme 73**. Only the reaction of (*S*)-**137** (A+B) is shown but all acylated dyes (*S*) and (*R*) **137-140** (A+B) were reacted. For clarity the approriate ester generated from each *N*-acyl dye is shown in **Table 13**. The yields obtained and the ratios of each ester obtained are documented in **Table 14**.

N-Acyl dye(A+B)	Ester
(*S*)-**137**	(*S*)-**141 A, B and C**
(*R*)-**137**	(*R*)-**141 A, B and C**
(*S*)-**138**	(*S*)-**142 A, B and C**
(*R*)-**138**	(*R*)-**142 A, B and C**
(*S*)-**139**	(*S*)-**143 A, B and C**
(*R*)-**139**	(*R*)-**143 A, B and C**
(*S*)-**140**	(*S*)-**144 A, B and C**

Table 13: *Ester compounds generated from each N-acyl dye*



Scheme 73: *Reagents and conditions* i. CH_3CN, K_2CO_3, formaldehyde

Ester	Yield (A+B)	Yield C	Yield (A+B+C)	Ratio (A+B):C	Isolation of A from B
(*S*)-**141**	33%	3.2%	36.2%	~10:1	√
(*R*)-**141**	31%	2.9%	33.9%	~11:1	√
(*S*)-**142**	51%	7.5%	58.3%	~7:1	X
(*R*)-**142**	48%	4.2%	52.2%	~11:1	X
(*S*)-**143**	82%	7.6%	88.6%	~11:1	√
(*R*)-**143**	67%	6.7%	73.7%	~10:1	√
(*S*)-**144**	65%	10%	75%	~7:1	X

Table 14: *Yields and ratios of esters A,B and C*

For all dyes, isolation of regioisomer **C** was easily achieved by column chromatography on silica gel, however isolation of **A** from **B** could only be achieved by preparative scale HPLC. Full experimental details are documented in **Chapter 5**. The regioisomers (*S*)-**142A+B**, (*R*)-**142A+B** and (*S*)-**144A+B** were inseparable by preparative HPLC despite extensive method development. The yield of esters (A+B), C and a combined yield (A+B+C) are documented in **Table 14**. The ratio of (A+B): C is also documented. Due to the small scale of these reactions and the problematic separation of A from B, the overall ratio A: B: C was not calculated. Full experimental yields and ratios of A: B are given in **Chapter 5.**

3.2.3 Structural determination of ester regioisomers A, B and C

The structure of the regioisomers **A**, **B** and **C** were confirmed by NMR and nuclear Overhauser enhancement (nOe) experiments. (*S*) and (*R*)-**141C** to **144C** were easily identified by comparison of the resonance obtained for the $\underline{C}H_2O$ peak in ^{13}C NMR with that obtained for **108** *i.e.* **144C** gave a signal at 75.4 compared to 74.9 for **108** while **141A** + **141B** gave 67.6 p.p.m and 67.4 p.p.m respectively. nOe spectroscopy confirmed the identity of (*S*)-**141A**, (*S*)-**141B** and (*S*)-**141C** and the results are displayed in **Figure 25.** This data was used to confirm the identity of the (*R*)- enantiomers.

The identity of (*S*)-**143A** was confirmed by COSY and nOe spectroscopy with irradiation at the frequency of the CH_2O giving an enhancement of 3.5% at the 7 position of the benzotriazole ring. In addition, irradiation of the CH_2O group gave no enhancement at the isolated benzotriazole proton – **Figure 26**. (Note (*S*)-143B did not give clear signals for any of the benzotriazole ring protons).

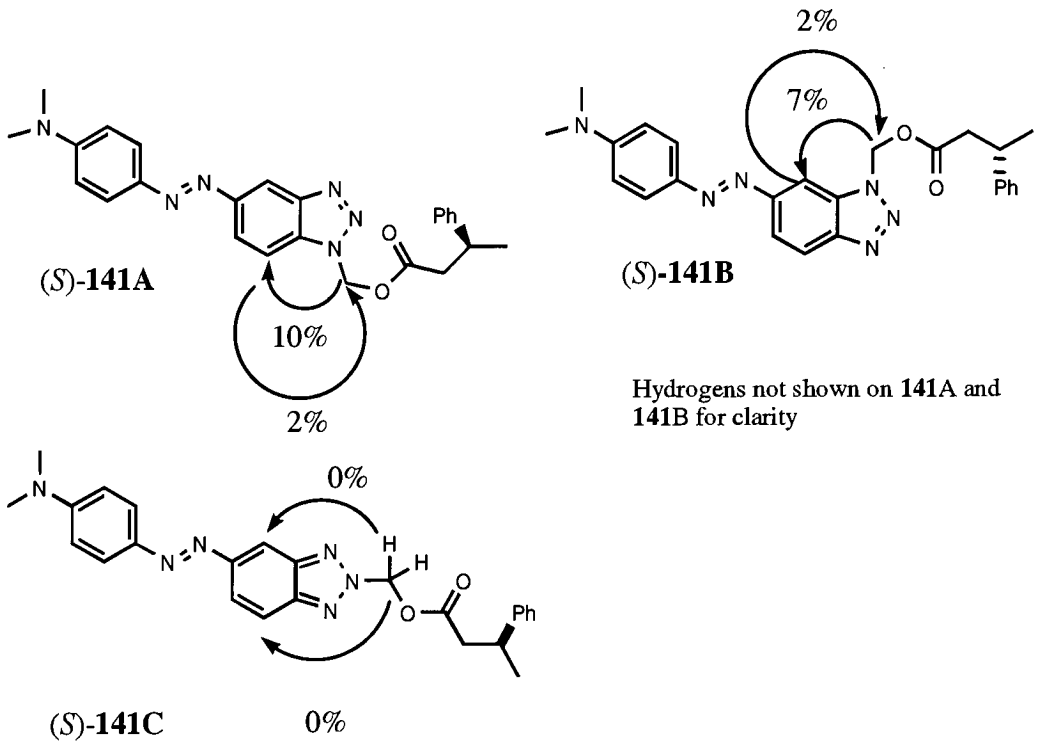


Figure 25: *nOe results for (S)*-**141A, B** and **C**

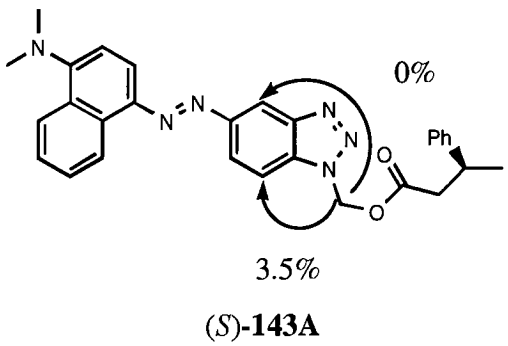


Figure 26: *nOe results for (S)*-**143A**

Measurement of the α_D values for these compounds was hampered by their strong absorbance at 589nm. The chiral integrity of the esters was established by cleavage using 1M NaOH and analysis by Chiral HPLC. The chromatograms obtained showed no racemization had occurred for either enantiomer of the esters **141-144** (**A, B** and **C**)

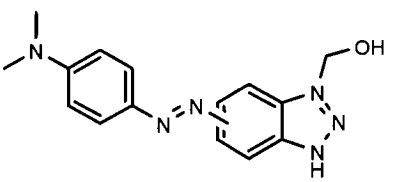
3.2.4 Summary

A wide range of phenolic, *N'N* dimethylaniline and pyrollidine monoazo-benzotriazole dyes **119-120** and **121-124** were produced. The crystal structure of **122** was obtained. The dyes (**121-124**) were successfully acylated with both (*R*) and (*S*) enantiomers of 3-phenylbutyryl chloride to generate both regioisomers of the *N*-acylated dye. In the case of (*S*)-**137A** and (*S*)-**137B** the regioisomers were separated by preparative scale HPLC and the crystal structure obtained for (*S*)-**137A**. These acyl derivatives were transformed into esters by the action of formaldehyde to generate three regioisomers and these were fully characterized by spectral analysis. Preparative scale HPLC and column chromatography were used to separate all three regioisomers of (*S*) and (*R*) **-141 (A-C)** and (*S*) and (*R*)-**143 (A-C)**. Chiral HPLC indicated that chiral integrity had been maintained throughout the synthesis.

3.2.4 Future Work

The generation and characterization of the 'ester' dyes was successful although separation of the regioisomers was protracted and final yields were low. There are several avenues that could be explored.

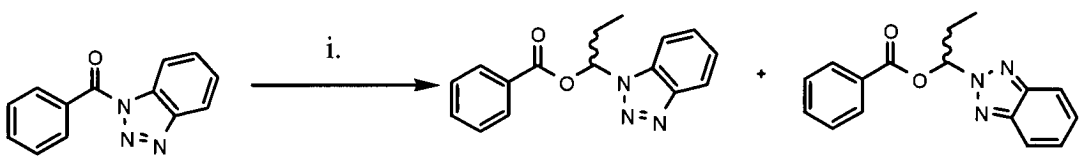
The 1-hydroxymethyl derivative *e.g.* **147** (shown for **121** only) of the nitrogen dyes **121-124** could be investigated and this may prove more fruitful than the corresponding compounds made from the phenolic dyes. If these can be successfully prepared then it should be possible to extend the chemistry to the preparation of esters of acids with α–chiral centres or of amino acids. This would allow much easier synthesis of the esters and allow more variation of the acid component.



147

3.3 Further studies on the synthesis of 2' benzotriazole esters.

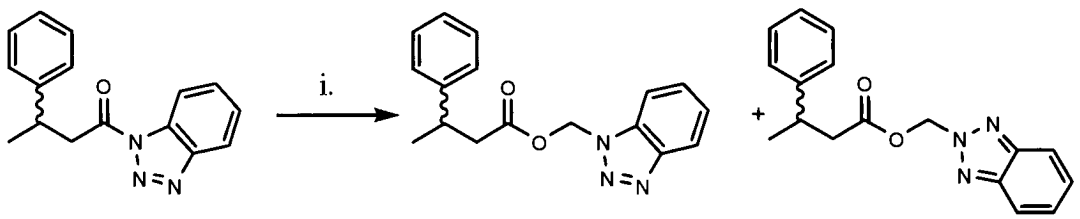
During investigations into the formation of the SERRS blocked dyes **141-144** it became evident that the separation of regioisomer **C** from the other regioisomers was much more straightforward *i.e.* it could be easily achieved by column chromatography without the need for prep-scale HPLC. It was felt that it might be possible to optimise the formation of this regioisomer, over the other two regioisomers, by careful control of the reaction conditions. Previously Katritzky *et al.*[137] had reported that the ratio of 1-(benzotriazol-1-yl)alkyl esters to 1-(benzotriazol-2-yl)alkyl esters could be influenced by the base and reaction solvent used. The ratio of **148**:**149** varied from 2.6:1 ($K^{+t}BuO^-$ in CH_3CN, 47% yield) to 13:1 (K_2CO_3 in hexane, 98% yield) for the reaction of 1-benztriazol-1-yl (phenyl) methanone **150** with propionaldehyde - **Scheme 74.**



150 **148** **149**

Scheme 74: *Reagents and conditions*: i. various bases, solvents, $CH_3CH_2CH{=}O$

With this in mind it was decided to investigate the reaction more fully and attempt to optimise the formation of the 2' isomer, in preference to the other regioisomer. In order to do this, the model reaction between (*R/S*)-**107** with formaldehyde to produce (*R/S*)-**105** and (*R/S*)-**108** was performed using a range of different reaction solvents and bases - **Scheme75**.



(*R/S*)-**107** (*R/S*) -**105** (*R/S*)-**108**

Scheme 75: *Reagents and conditions:* i. base, solvent, formaldehyde, RT

3.3.1 Studies on the influence of base concentration

Potassium carbonate and triethylamine have been shown[137] to give excellent yields, 100% and 86% respectively, of 1-(1*H*-benzotriazol)propyl benzoate **148+149** however the ratio of **148**:**149** varied from 4:1 to 10:1, respectively – **Scheme 74**. These two bases were the starting point for our study. Each base was used at a concentration of 5, 15 or 25 mol%, with respect to the *N*-acylbenzotriazole **107,** to investigate its effect on the ratio **105**:**108.** Small scale (0.4mmol) reactions were performed using a Gilson SK233 system and the reactions monitored by HPLC every hour for 5hours and then again after 24 hours – **Table 15**. The 24hour time point allowed us to monitor slower reactions and to confirm that **108** was stable throughout the course of the reaction.

Base	105 (5hr)	108 (5hr)	105 (24hr)	108 (24hr)	Ratio 1:2 (24hr)
5% K_2CO_3	1.2%	N/D	44.7%	0.7%	64:1
15% K_2CO_3	66.7%	1.6%	70.3%*	**1.7%**	**41:1**
25% K_2CO_3	68.7%	1.5%	71.1%*	1.5%	47:1
5% Et_3N	0.5%	-	8.5%	-	-
15% Et_3N	36.8%	0.4%	46.2%	0.6%	77:1
25% Et_3N	43.7%	0.5%	54.5%	0.8%	68:1

Table 15 – *Effect of varying concentration of K_2CO_3 and Et_3N (5 and 24 hour results)*

The results are similar to those obtained by Katritzky[137] with triethylamine giving markedly lower combined yields of the esters and a higher ratio of **105**:**108** ester than K_2CO_3. Under all reaction conditions with K_2CO_3, the ratio of **105**:**108** esters was significantly in favour of **105**, much more so than that which had been reported for the propionaldehyde esters. This phenomenon had been observed previously during the preparative scale reactions and discussed in **Section 2.1.2**. The ratio observed by HPLC was much higher than that observed from the prep-scale reactions but only small amounts of **108** were ever isolated and the HPLC results are area% only.

This series of experiments did identify that 15 mol% was the optimum concentration of base required to maximise both the combined yield and the yield of the 2' ester. This concentration of base was used to study the effect other bases, both organic and inorganic, had on the formation of ester **108**.

3.3.2 Studies on the influence of different bases

A range of different organic and inorganic bases were used in the reaction displayed - **Scheme 75**. All were used at the optimal concentration of 15 mol % in CH_3CN. Again the reactions were analysed by HPLC every hour for five hours and then again at 24hours. The results are displayed - **Table 16**.

K^+t-BuO^-, DMAP and DBU all give comparable yields to K_2CO_3 with K^+t-BuO^-, giving an increased yield of ester **108**. This is in agreement with the results of Katritzky et al[137] who reported a ratio of 2.6:1 (**148**:**149**, 47% yield) for K^+t-BuO^- and a reaction time of 0.25hrs. In addition, Chiral HPLC analyses of the reaction mixture indicated no racemisation of **105** using K^+t-BuO^-.

In these small-scale experiments the reactions were complete (within experimental error) in under 5 hours for the aforementioned three bases. Na_2CO_3 gave the highest yield of product and a slightly increased yield of the **108** but the reaction was slower and remained incomplete after 48 hours.

Base (15 mol %)	Area% 105 (5hr)	Area% 108 (5hr)	Area% 105 (24hr)	Area% 108 (24hr)	Ratio 105:108
Na_2CO_3	16.8	0.6	76.9*	**3.1***	**25:1**
Li_2CO_3	0.7	N/D	4.4	N/D	-
$CaCO_3$	0.5	N/D	0.7	N/D	-
KH_2PO_4	**No Reaction**				
K^+t-BuO^-	68.7	2.7	68.9	**2.9**	**24:1**
Pyridine	0.8	N/D	1.0	N/D	-
N-Ethyl Morpholine	0.6	N/D	10.0*	N/D	-
Cyclohexylamine	3.0	N/D	34.9*	0.5%	69:1
Morpholine	5.7	N/D			
DMAP	66.9	1.2	70.2	1.3	54:1
DBU	76.0	1.2	76.0	1.3	59:1
DIPEA	**No Reaction**				
Guanidine	**No Reaction**				
Imidazole	0.6	N/D	0.3	N/D	-

Table 16 – *Effect of base on the ratio of* **105:108** (**denotes 48 hour time point*)

3.3.3 Studies on the influence of different solvents

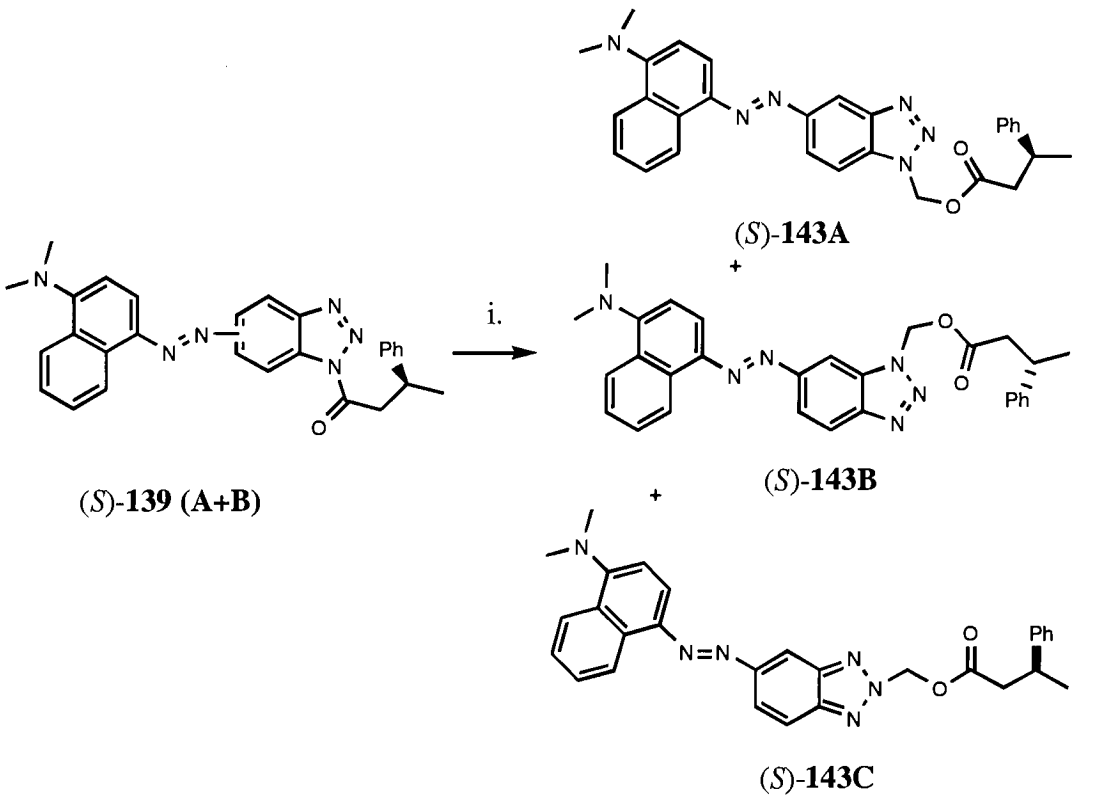
The reaction in **Scheme 75** was further studied using 15mol% K_2CO_3 in a range of polar and non-polar solvents and the results displayed - **Table 17**. All the solvents studied gave much lower yields of **105** and **108** compared to acetonitrile and the ratios **105**:**108** are skewed because the reaction has not gone to completion. The breakdown of the starting material in MeOH, formaldehyde and DMF is probably due to the presence of competing nucleophiles displacing the benzotriazole from **107**.

Solvent	105 (5hr)	108 (5hr)	105 (24hr)	108 (24hr)	Ratio 105:108 (24hr)
DCM	1.1	N/D	21.3	0.9	24:1
EtOAc	2.2	N/D	20.3	0.8	25:1
THF	7.8	0.5	7.1	0.5	14:1
Formaldehyde	breakdown to benzotriazole				
IPA	4.3	N/D	27.6	0.6	46:1
MeOH	breakdown to benzotriazole				
DMF	breakdown to benzotriazole				
DMSO	41.4	1.0	44.3	1.2	39:1
t-Butyl methyl ether	3.2	N/D	4.5	N/D	-
1,4 Dioxane	No Reaction				
2,2 Dimethoxypropane	19.2	0.5	38.7	0.7	55:1

Table 17 – *Effect of solvent on ratio of* **105**:**108**

3.3.4 Application to SERRS dyes

To complete this study (*S*)-**139 (A+B)** was reacted with formaldehyde in the presence of K^+t-BuO^- to determine if the yield of (*S*)-**143C** was increased – **Scheme 76**. Using these conditions, the yield of (*S*)-**143(A+B)** was 79% and the yield of (*S*)-**143C** was 8%, giving a ratio of ~10:1. This was identical to that obtained using K_2CO_3 indicating that further studies using the *N*-acyl SERRS dyes are required and may prove more informative.



Scheme 76: *Reagents and conditions* i. formaldehyde, CH_3CN, K^+t-BuO^-, RT

3.3.5 Summary

In conclusion, limited success was achieved with the reaction optimization studies. Four bases (Na_2CO_3, K^+t-BuO^-, DBU and DMAP) were identified which could potentially increase the yield of ester **108**, although only small changes were observed. The use of K^+t-BuO^-, did not result in the increase of regioisomer **C** when applied to *N*-acyl SERRS dye (*S*)-**139(A+B)**. Changing the solvent from CH_3CN had a severely detrimental effect on the yield of the reaction.

Chapter 4 – Results and Discussion III

4.1 Preliminary SERRS results

Following the successful synthesis and isolation of the blocked SERRS dyes (*S*) and (*R*) **141-144** (**A**, **B** and **C**), these and the parent dyes **121-124** were supplied to the laboratories of Dr Duncan Graham and Dr Barrie Moore, at the University of Strathclyde, for analysis by Lorna Stevenson using SERRS. Unfortunately, at the time of writing only limited data is available.

4.1.1 SERRS analysis of dyes 121-124.

As mentioned previously, for SERRS determination of enantioselectivity to be successful the dyes must be able to be distinguished from each other and the amount of each dye quantified without a separation step. In addition, the 'blocked' SERRS dyes **141-144** (**A, B** and **C**) must remain SERRS inactive until the parent dye is released by enzymatic hydrolysis.

Firstly, the dyes were analysed individually to determine if they were SERRS active and provided a measurable SERRS spectra at the chosen excitation wavelength (514nm) and concentration (10^{-7}M). A typical procedure for measuring SERRS spectra is documented in **Chapter 5**. The spectra obtained for dye **121** is shown - **Figure 27.** The most intense peak at ~ 1400cm^{-1} is attributed to stretching of the azo bond. The spectra obtained for dyes **121**, **122** and **123** are contained within **Appendix 7.3.** At present, no SERRS spectrum is available for dye **124** and the limit of quantification of dyes **121-123** has not been established.

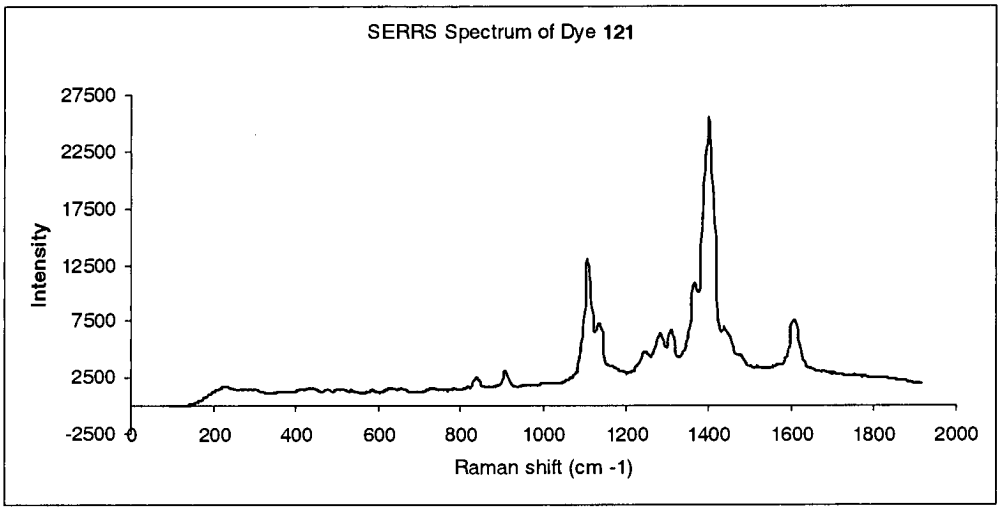


Figure 27: *SERRS spectra of Dye* **121**

4.1.2 SERRS quantification of mixtures of dyes 121 and 122.

A regression model was built by analysing known mixtures of dyes **121** and **122** (*i.e.* using solutions ranging from 100% **121** / 0% **122** to 0% **121** / 100% **122**). This model was then used to predict, using a variety of processing methods, a known concentration of dye **121** in the presence of dye **122** – **Table 18**.

Data processing method	**Known** vs. Predicted composition			
	12.5% - 121	**33% - 121**	**67% - 121**	**87.5% -121**
Raw spectra – full range	38.2	48.4	46.6	47.6
Raw spectra - (1000-1800 cm^{-1})	37.1	48.3	45.7	47.0
1^{st}derivative – full range	36.4	26.3	43.9	46.3
1^{st} derivative – (1000-1800 cm^{-1})	28.9	45.2	41.9	54.6
1^{st} derivative – (1300-1450 cm^{-1})	35.5	41.8	48.7	56.1
1^{st} derivative – (1450-1700 cm^{-1})	34.0	19.9	47.1	55.7

Table 18: *Known vs Predicted compositon of* **121** (results in bold are actual composition)

The results displayed - **Table 18** indicate that quantification of dye **121** in the presence of **122** was not possible using SERRS analysis. The reason for this remains unknown although several possible explainations have been suggested – *e.g.* variability of spectra with time due to changes in level of aggregation or the spectrum from each dye is not significantly distinct from each other (a comparison of the spectrum from **121** with that from **122** is contained within **Appendix 7.3**). At the time of writing, investigations into the variability of SERRS spectra with time and degree of aggregation are continuing. It is hoped that continuous flow analysis may reduce the inherent variability of each spectrum. Further investigations are also underway into the quantification of dye **121** in the presence of dye **123**.

4.1.3 SERRS analysis of ester 141 (A).

A 10^{-6}M solution of (*S*)-ester **141(A)** was analysed by SERRS using an excitation wavelength of 514 nm – **Figure 28**.

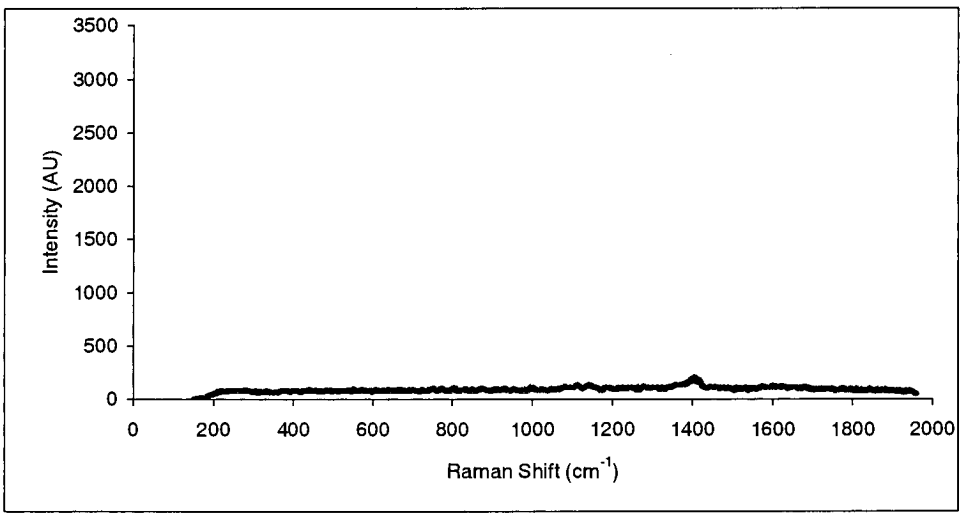


Figure 28: *SERRS spectra of ester* **141A**

It is clear from **Figure 28** that ester **141A** does not display a SERRS spectra similar to the parent dye **121.** It must be noted however, that the order of addition of reagents altered the SERRS spectra observed. A simple buffered solution of ester **141A** and colloid gave a SERRS spectra but the addition of a protein, prior to addition of the

ester gave the spectra displayed in **Figure 28**. This observation suggested that a non-specific interaction, *i.e.* not through the nitrogens of the benzotriazole ring, between **141A** and the silver colloid was occurring. It was suggested that this might be due to the *N'N'* -dimethylaniline moeity of the compound, a feature common to esters **141-143 (A-C).** Following this observation dye **124**, replacing the *N'N'*-dimethyl moiety with a pyrollidine ring, was synthesized and converted to the esters **144(A-C)**. Unfortunately at present no data is available to determine if this has solved the problem. Further studies are also continuing into the use of other additives to reduce the SERRS activity of the 'blocked' dyes.

4.1.4 Treatment of ester 141 (A) with lipase.

Into a buffered solution of colloid and *Pseudomonas cepacia* lipase (with the lipase acting as the protein additive) was added a solution of ester (*S*)- **141A**. The SERRS spectrum of the solution was measured at time zero and after 895 seconds – **Figure 29**.

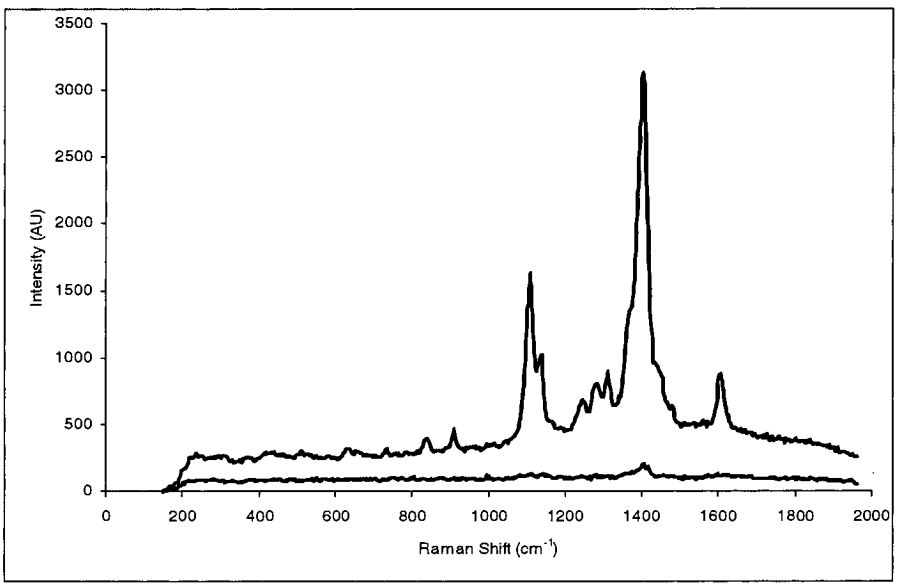


Figure 29: *SERRS spectra of ester* **141A** *at zero (bottom) and 895 seconds (top) with PCL*

Figure 29 clearly shows that ester (*S*)-**141A** has been hydrolysed to release the parent dye **121.** This was confirmed by monitoring the reaction by HPLC. The intensity of the SERRS signal increased with time indicating that the concentration of dye **121** present was also increasing with time. A control reaction, with no lipase present, did not show the appearance of the dye SERRS spectra.

Following the successful monitoring of an enzyme reaction using SERRS we compared the rate of enzymatic hydrolysis of (*R*)-**141A** with that of (*S*)-**141A** using *Pseudomonas cepacia* lipase (PCL) and *Candida antarctica* lipase (CAL). PCL is known to hydrolyse (*S*)-**105** more rapidly than the (*R*) enantiomer (E = 11.7, **Section 2.2.2**). The results obtained are displayed in **Figure 30** and **Figure 31**. Each figure shows the increase in intensity of the stretch at 1407cm^{-1} with time.

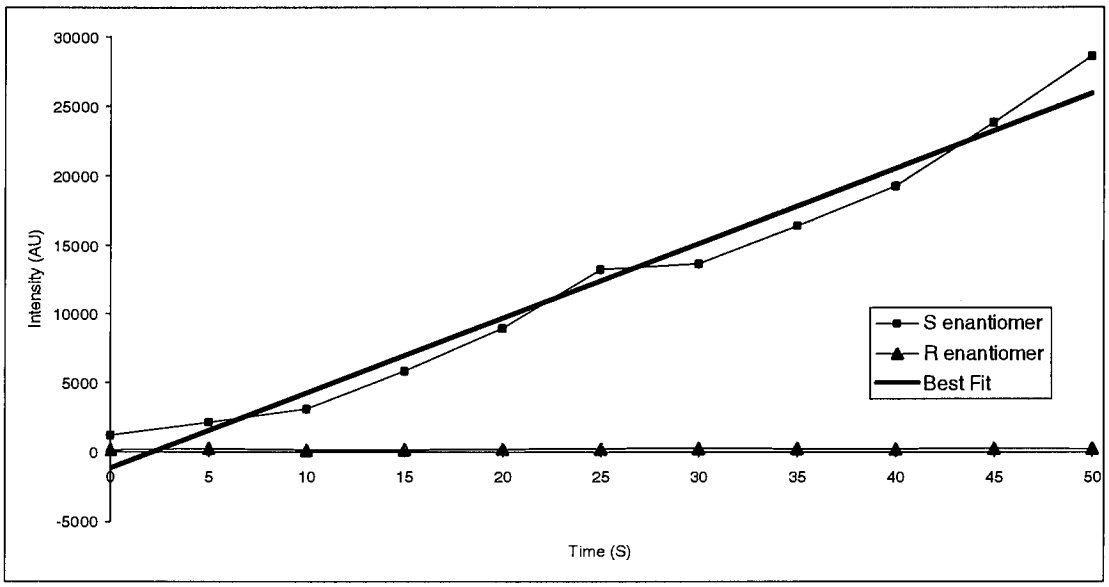


Figure 30: *Comparison of hydrolysis rates of* (*S*) *and* (*R*)-**141A** *using PCL*

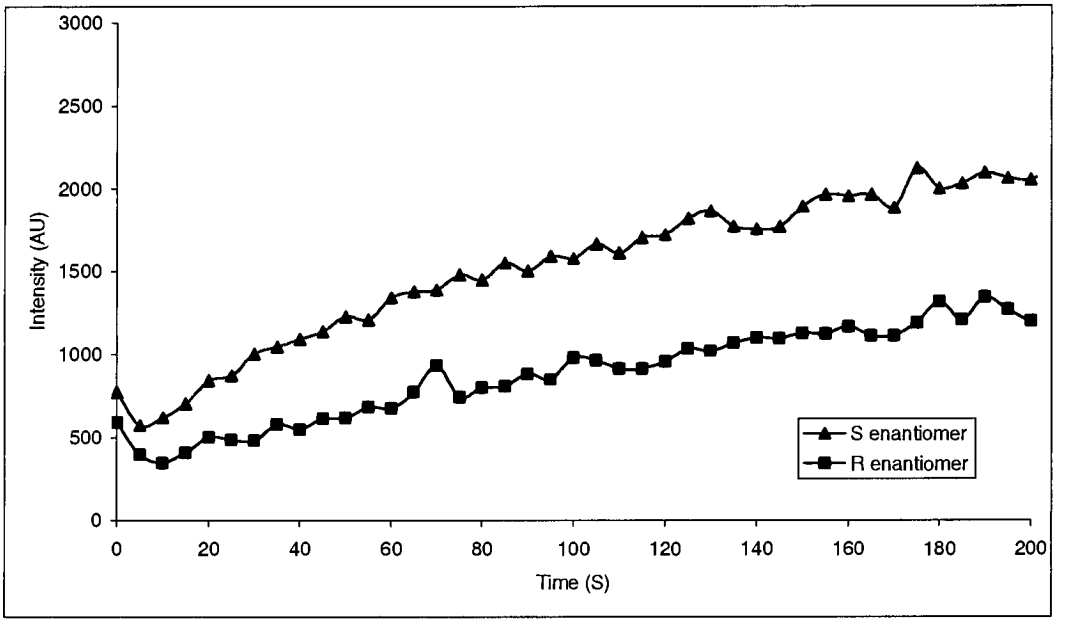


Figure 31: *Comparsion of hydrolysis rates of (S) and (R)* **-141A** *using CAL*

Comparison of the absolute intensity of the signal generated during PCL and CAL hydrolysis clearly indicates that PCL hydrolyses the ester more rapidly. Unfortunately, this series of experiments was carried out using unknown concentrations of (*S*) and (*R*)-**141A,** making determination of the *E* value impossible. However, the concentration of the esters was constant between each different enzyme indicating that PCL is qualitatively more enantioselective than CAL, prefering to hydrolyse the (*S*) enantiomer of **141A** more rapidly.

4.1.5 Future Work

At the time of writing investigations are continuing into several areas concerning the analysis of enzymatic reactions using SERRS. These include the use of various additives to reduce the SERRS signal for the blocked esters, investigations into the quantification of mixtures of dyes and studies on controlling the reproducibility of SERRS spectra using different colloids and continuous flow cell measurements.

Chapter 5 - Experimental

5.1. General Experimental

5.1.1. Instrumentation

^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker AC200, Bruker AC250 Varian Gemini 200 and Varian Inova 600 instruments. Chemical shifts (δ_H, δ_c) are reported in p.p.m. and coupling constants (J) are in Hertz (Hz).

Electrospray (ES) mass spectrometry was performed using a Micromass platform II instrument with Mass Lynx software. Fast Atom Bombardment (FAB) mass spectrometry was performed using a Kratos MS50TC instrument.

Infrared spectroscopy was performed using a Perkin Elmer Paragon 1000 FT-IR spectrometer with the frequencies (ν) measured in wavenumbers (cm^{-1}). Samples were measured on disposable IR cards (Type 61 3M, polythene, 19mm aperture), in DCM as thin films, neat or as nujol mulls.

Experimental design experiments were carried out on an Anachem SK233 system and analysed by reverse phase HPLC.

Optical rotations were measured on an Optical Activity AA-1000 polarimeter (sodium 589nm detection). Sample concentration was measured in g/100ml and $[\alpha]^{20}_{D}$ values are quoted in 10^{-1} deg cm^{-1}.

Melting points were recorded on a Gallenkamp melting point apparatus and were uncorrected.

Elemental analysis (CHN) was performed on a Perkin Elmer 2400 CHN Elemental Analyser.

A 360° Stuart scientific SB1 blood rotator was used for enzymatic hydrolysis experiments.

5.1.2. Chromatography

Analytical thin layer chromatography (TLC) was performed using aluminium-backed plates coated with silica gel $60F_{254}$ (Merck: layer thickness of 0.2 mm).

Identification was carried out using U.V. fluorescence (254nm), *p*-anisaldehyde, ammonium molybdate, potassium permanganate and ninhydrin dips. Flash column chromatography was carried out with a variety of columns using BDH silica gel (40-63μm) or a Biotage pre-packed cartridge system.

5.1.3. High Pressure Liquid Chromatography

Analytical high-pressure liquid chromatography (HPLC) was performed using a Waters tuneable 486 Absorbance Detector, Waters autosampler and a Waters 600E pump and controller. These were managed by the Waters Millennium Chromatography software package. A Phenomenex Sphereclone 25cm x 0.46 cm 5μm ODS 2 column was used for all reverse phase HPLC and either a Diacel Industries Chiracel OD 25cm x 0.46cm, OD-H 25cm x 0.46cm or Chiralpak AD 25cm x 0.46cm column was used for all normal phase chiral HPLC. In all cases, a flow rate of 1ml/min was used and detection was at 210nm for reverse phase and 254nm for normal phase chromatography. Due to the number of methods developed the exact mobile phase composition and column temperature is documented within the analytical data for the specific compound of interest. Retention times (R_t) are quoted in minutes.

For quantitative analyses, two concordant standards bracketed the samples or the relative response factor of each analyte with respect to the main peak was calculated. For qualitative analyses results are quoted on an Area% basis.

5.1.4. Preparative High Pressure Liquid Chromatography

Preparative scale HPLC was performed on a Biotage FLEX system using FLEX control software. 2 columns were used depending on the amount of sample to be purified; less than 40mg a Supelcosil ABZ+ 5cm x 21.2mm i.d. with 0.5ml injection volume was used and greater than 40mg a Supelcosil ABZ+ 25cm x 21.2mm i.d. with 1ml injection volume. A flow rate of 20ml/min at ambient temperature was used for both columns and detection was by UV absorbance at 254 and 220nm. Due to the number of

methods developed the exact mobile phase composition is documented within the analytical data for the specific compound of interest. Fractions were collected into plastic 48 or 96 well plates, carefully pooled and subjected to further purification if required.

5.1.5. Solvents and Reagents

All reagents were standard laboratory grade and used as supplied unless otherwise stated. For HPLC methods: UV grade acetonitrile, distilled water, HPLC grade *iso*-propanol, hexane and trifluoroacetic acid were used. Dichloromethane was distilled from calcium hydride, while tetrahydrofuran was pre-dried over sodium wire and distilled from sodium benzophenone ketal. All other 'dry' solvents were purchased as anhydrous grade.

5.1.6. Enzymes

All enzymes were purchased from Sigma, Fluka (from a Basic Lipase or Esterase kit) or Biocatalysts and the specific activities are documented below. α-Chymotrypsin was purchased from Sigma as a three times recrystallised lyophilised powder with an activity of 40U/mg of solid.

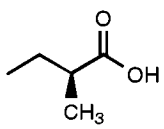
Lipase	Activity	Esterase	Activity
Pseudomonas cepacia	40U/mg	*Bacillus sp.*	0.15U/mg
Porcine pancreatic	16.8U/mg	*Bacillus stearthermophillus*	0.41U/mg
Aspergillus niger	1.4U/mg	*Bacillus thermoglucosidasius*	0.082U/mg
Mucor miehei	1.3U/mg	*Candida lipolytica*	0.1U/mg
Pseudomonas. fluorescens	42.5U/mg	*Mucor miehei*	1.2U/mg
Rhizopus. niveus	2.6U/g	Horse Liver	0.96U/mg
Rhizopus. arrihzus	2U/g	*Saccharomyces cerevisiae*	2.55U/g
Candida. antarctica	2.9U/mg	Hog Liver	251.0U/mg
Candida cylindrecea	2.4U/mg	*Thermoanaerobium brockii*	4.9U/g
Mucor. javanicus	5.2U/mg		

5.1.7 SERRS instrumentation

All SERRS measurements were performed by Lorna Stevenson at the University of Strathclyde using a Reinshaw Mark III probe system (Reinshaw Ltd, UK) with a Spectra Physics 362C 15mW-argon ion laser. All spectra were collected using a excitaition wavelength of 514.5nm. Silver collid solutions were prepared using a modified Lee and Meisel procedure.[124]

5.2.0 Preparation of benzotriazole containing substrates

5.2.1 (*S*)-2-methyl butyric acid (102)



(*S*)-2-Methyl butan-1-ol (5g, 57mmols) and sodium carbonate (1.22g, 15mmols) were dissolved in 6ml of H_2O and cooled to 0°C in an ice bath. The resulting solution was stirred vigorously and potassium permanganate (11.5g, 73mmols) dissolved in H_2O (220ml) was added dropwise over a period of 4 hours. The reaction mixture was allowed to stir overnight at room temperature. The black precipitate was filtered off and the solution concentrated to small volume under reduced pressure. The remaining aqueous solution was acidified with H_2SO_4 (1M) and the aqueous layer extracted with ether (250ml). The organic layer was separated, dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to give a clear liquid (3.7g, 64%). The liquid was further purified by distillation to give the desired product as a clear liquid (3.3g, 57%).

Bp 54°C at 1mbar, Lit[141] Bp 50-52°C at 0.7torr; υ_{max}(nujol)/cm^{-1} 3091 broad to 2565 (OH) , 2969, 2936, 2878 (CH), 1706 (C=O) ; δ_H ($CDCl_3$; 250MHz) 0.93 (3H, t, J 7.4Hz, $C\underline{H}_3$), 1.16 (3H, d, J 6.9Hz, $C\underline{H}_3$), 1.43-1.57 (1H, m, $C\underline{H}_aH_bCH_3$), 1.61-1.79 (1H, m, $CH_a\underline{H}_bCH_3$) and 2.41 (1H, tq, 7.0 and 7.0Hz, $CH_2C\underline{H}C=O$); δ_C ($CDCl_3$; 63MHz) 11.0

($CH_2\underline{C}H_3$), 14.6 ($CH\underline{C}H_3$), 24.8 (CH_2), 43.3 ($\underline{C}H$) and 179.6 (C=O); $[\alpha]_D^{20}$ +19.3 (1.44, $CHCl_3$), Lit[141] $[\alpha]_D^{20}$ +19.6 (1.15, $CHCl_3$).

5.2.2 General Procedure for preparation of acid chlorides

Two methods were employed for the conversion of the carboxylic acid to acid chloride. For small amounts of acid, oxalyl chloride was used and for large scale (>5g) conversion thionyl chloride was employed.

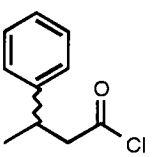
Thionyl Chloride method:

The appropriate acid (1 equiv.) was refluxed neat with an excess of thionyl chloride and a catalytic amount of DMF for 2 hours (or until conversion to the acid chloride was complete as determined by IR spectroscopy). The unreacted thionyl chloride was removed by distillation and the acid chloride purified by bulb to bulb kegelrohr distillation under reduced pressure (if required).

Oxalyl Chloride method:

The acid (1 equiv.) was stirred at room temperature with oxalyl chloride (1.5 equiv.) and a catalytic amount of DMF (typically 2-3 drops) for 2 hours (or until conversion to the acid chloride was complete as determined by IR spectroscopy). The acid chloride was purified by bulb-to-bulb kegelrohr distillation under reduced pressure (if required.)

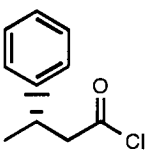
5.2.3 (*R/S*)-3-Phenyl-butyryl chloride (107)



The general procedure (**5.2.2**) was followed with (*R/S*)-3-phenylbutyric acid (6.56g, 4mmols), thionyl chloride (1.5 equivs, 4.32ml, and 6mmols) and catalytic amount of DMF to provide the desired product as a moisture sensitive clear liquid (5.99g, 82%).

Bp 100°C at 0.5mbar, Lit[165] Bp 104 at 0.4mbar; υ_{max}(neat)/cm^{-1} 3029 (CH_{ar}), 2968, 2936, 2878 (CH), 1801 (C=O), 1604 (C=C), 1495, 1453 (C=C); δ_H ($CDCl_3$; 250MHz) 1.36 (3H, d, J 6.9Hz, $C\underline{H}_3$), 3.13 (1H, dd, J 16.5 and 8Hz, $C\underline{H}_aH_bCH_3$), 3.19 (1H, dd, J 16.5 and 6.5Hz $CH_a\underline{H}_bCH_3$), 3.37 (1H, m, $C\underline{H}CH_3$) and 7.19-7.37 (5H, m, Ph); δ_C ($CDCl_3$; 63MHz) 21.8 ($\underline{C}H_3$), 37.1 ($\underline{C}H$), 55.5 ($\underline{C}H_2$), 127.1 ($\underline{C}H_{ar}$ x 2), 127.4 ($\underline{C}H_{ar}$), 129.2 ($\underline{C}H_{ar}$ x 2), 144.4 ($\underline{C}_{ar}$) and 172.8 (C=O).

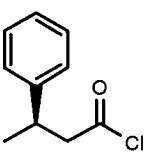
5.2.4 (*S*)-3-Phenyl-butyryl chloride (107)



The general procedure **5.2.2** was followed with (*S*)-3-phenylbutyric acid (2.5g, 15mmols), oxalyl chloride (1.5 equivs, 2.9g, 23mmols) and a catalytic amount of DMF to provide the desired product as a moisture sensitive clear liquid (2.47g, 88%).

Analysis comparable to **5.2.3** except; Bp 105°C at 0.4mbar, Lit[165] Bp 104°C at 0.4mbar; $[\alpha]_D^{20}$ +21.9 (1.2, $CHCl_3$), Lit $[\alpha]_D^{20}$ +21.5 (1.1, $CHCl_3$).

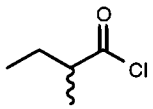
5.2.5 (*R*)-3-Phenyl butyryl chloride (107)



The general procedure **5.2.2** was followed with (*R*)-3-phenylbutyric acid (2.5g, 0.015mols), oxalyl chloride (1.5 equivs, 2.9g, 0.023mols) and a catalytic amount of DMF to provide the desired product as a moisture sensitive clear liquid (2.29g, 83%).

Analysis comparable to **5.2.3** except Bp 110°C at 0.6mbar, Lit[165] Bp 104°C at 0.4mbar; $[\alpha]_D^{20}$ –22.1 (1.2, $CHCl_3$), Lit $[\alpha]_D^{20}$ –22.0 (1.0, $CHCl_3$)

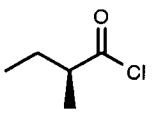
5.2.6 (*R/S*) 2-methyl-butyryl chloride (109)



The general procedure **5.2.2** was followed with commercially available (*R/S*)-2-methylbutyric acid (5g, 0.05mols), thionyl chloride (2 equivs, 7.7ml, and 0.065mols) and catalytic amount of DMF to provide the desired product as a moisture sensitive clear liquid (4g, 67%).

Bp 112-116°C, Lit[165] Bp 114-120°C; υ_{max}(neat)/cm^{-1} 2971, 2936, 2878 (CH), 1794 (C=O); δ_H ($CDCl_3$; 250MHz) 0.93 (3H, t, J 7.4Hz, $CH_2C\underline{H}_3$), 1.16 (3H, d, J 6.9Hz, $CHC\underline{H}_3$), 1.43-1.57 (1H, m, $C\underline{H}_aH_bCH_3$), 1.61-1.79 (1H, m, $CH_a\underline{H}_bCH_3$) and 2.39 (1H, tq, J 6.9 and 7.0Hz, $C\underline{H}CH_3$); δ_C ($CDCl_3$; 63MHz) 11.4 ($CH_2\underline{C}H_3$), 16.2 ($CHC\underline{H}_3$), 26.4 ($\underline{C}H_2$), 40.9 (CH), and 183.5 (C=O).

5.2.7 (*S*)-2-methyl-butyryl chloride (109)



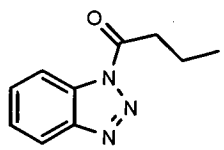
The general procedure **5.2.2** was followed with (*S*)-2-methylbutyric acid (*S*)-**102** (2.2g, 0.021mols), thionyl chloride (1.5 equivs, 3.78g, 0.032mols) and a catalytic amount of DMF to provide the desired product as a moisture sensitive clear liquid (1.96g, 76%).

Analysis comparable to (*R/S*)-**109** except Bp 114-116°C, Lit Bp[141] 110-116°C; $[\alpha]_D^{20}$ +10.5 (1.13, $CHCl_3$), Lit $[\alpha]_D^{26}$ +12.1 (neat).

5.2.8 General procedure for preparation of *N*-acyl benzotriazoles

Benzotriazole (1 equiv.) was dissolved in an appropriate volume of 'anhydrous' DCM under an inert atmosphere of nitrogen. The solution was cooled to 0°C in an ice bath and a solution of triethylamine (1.2 equiv.) in 'anhydrous' DCM was added dropwise followed by dropwise addition of a solution of the appropriate acid chloride (1.1 equiv.) in 'anhydrous' DCM. The solution was stirred at 0°C for 30mins then allowed to warm to room temperature and stirring continued for a further 1 hour. After this time the reaction was quenched by addition of HCl (2M) and the organic layer separated. The organic layer was washed with further volumes of HCl (2M), H_20, brine, then dried over $MgSO_4$, filtered and the organic solvent removed under reduced pressure to provide the desired acylated benzotriazole as the 1' isomer. Purification (if necessary) by column chromatography on silica gel or recrystallization was then performed.

5.2.9 1-Benzotriazol-1-yl-butan-1-one (92)

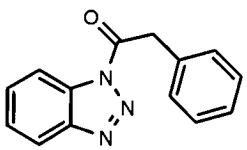


The general protocol **5.2.8** was followed with benzotriazole (10g, 0.084moles) dissolved in 'anhydrous' DCM (170ml) with the addition of triethylamine (1.2equivs., 14.3ml, 0.101mols) and butyryl chloride (1.1 equivs., 9.85g, 0.092mols). The desired product was isolated as an off white solid and recrystallised (from pentane) to give clear crystals. Note: The solid is unstable at room temperature releasing the very characteristic smelling butyric acid.

R_f (Hexane: EtOAc, 5:1) 0.52; Mp 58-60°C, Lit[166] 60-62; υ_{max} (nujol)/cm^{-1} 2968, 2914, 2881, 2852 (CH), 1738 (C=O), 1594 (C=C), 1482; δ_H ($CDCl_3$; 250MHz) 1.08 (3H, t, J

7.5Hz, $C\underline{H}_3CH_2$), 1.92 (2H, tq, J 7.5 and 7.5Hz, $CH_3C\underline{H}_2$), 3.38 (2H, t, J 7.5Hz, $C\underline{H}_2C{=}O$), 7.47 (1H, ddd, J 8.2, 7.1 and 1.1Hz, $C\underline{H}_{bt}$), 7.62 (1H, ddd, J 8.2, 7.1 and 1.1Hz, $C\underline{H}_{bt}$), 8.09 (1H, ddd, J 8.2, 1.1 and 1.0Hz, $C\underline{H}_{bt}$) and 8.26 (1H, ddd, J 8.2, 1.1 and 1.0Hz, $C\underline{H}_{bt}$); δ_H ($CDCl_3$; 63MHz) 13.5 ($\underline{C}H_3CH_2$), 17.8 ($CH_3\underline{C}H_2$), 37.2 ($\underline{C}H_2C{=}O$), 114.3 ($\underline{C}H_{bt}$), 119.9 ($\underline{C}H_{bt}$), 125.9 ($\underline{C}H_{bt}$), 130.2 ($\underline{C}H_{bt}$), 130.9 ($\underline{C}_{bt}$), 146.0 ($\underline{C}_{bt}$) and 172.4 (C=O); m/z (ESi +ve, 45kV) 190 (100%, $[MH]^+$); m/z (FAB, 3-NOBA) 190 (90%, $[MH]^+$), 162 (65%, $[MH\text{-}CH_2CH_3]^+$), 146 (60%, $[MH\text{-}CH_2CH_2CH_3]^+$), 120 (100%, $[BtH]^+$) and 77 (45%, $[Ph]^+$); m/z (FAB, 3-NOBA) Found 190.098037 $C_{10}H_{12}N_3O$ requires 190.09775.

5.2.10 1-Benzotriazol-1-yl-2-phenyl-ethanone (93)

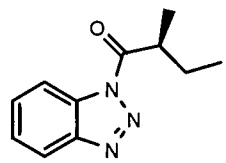


The general protocol **5.2.8** was followed with benzotriazole (2.98g, 25mmols, 1 equiv.) dissolved in 'anhydrous' DCM (50ml) with the dropwise addition of triethylamine (4.25ml, 30mmols, 1.2 equiv.) and phenacetyl chloride (4.25g, 27.5mmols, 1.1 equiv.) Purification by column chromatography on silica gel eluting with DCM provided the desired product as a pale yellow oil (4.37g, 78%).

R_f (DCM) 0.80; υ_{max} (nujol)/cm^{-1} 2968, 2914, 2881, 2852 (CH), 1740 (C=O), 1594 (C=C), 1482; δ_H ($CDCl_3$; 250MHz) 4.72 (2H, s, $C\underline{H}_2C{=}O$), 7.24-7.45 (5H, m, $C\underline{H}_{ar}$ x 5), 7.49 (1H, ddd, J 8.3, 7.2 and 1.1Hz, $C\underline{H}_{bt}$), 7.62 (1H, ddd, J 8.3, 7.2 and 1.1Hz, $C\underline{H}_{bt}$), 8.11 (1H, ddd, J 8.2, 1.0 and 1.0Hz, $C\underline{H}_{bt}$) and 8.25 (1H, ddd, J 8.2, 1.1 and 1.0Hz, $C\underline{H}_{bt}$); δ_H ($CDCl_3$; 63MHz) 41.2 ($\underline{C}H_2C{=}O$), 114.3 ($\underline{C}H_{bt}$), 120.0 ($\underline{C}H_{bt}$), 126.1 ($\underline{C}H_{bt}$), 127.5 ($\underline{C}H_{ar}$), 128.7 ($\underline{C}H_{ar}$ x 2), 129.7 ($\underline{C}H_{ar}$ x 2), 130.3 ($\underline{C}H_{bt}$), 131.1 ($\underline{C}_{ar}$), 132.2 ($\underline{C}_{bt}$), 146.1 ($\underline{C}_{bt}$) and 170.1 (C=O); m/z (ESi +ve, 45kV) 238 (100%, $[MH]^+$); m/z (FAB, 3-

NOBA) 238 (90%, $[MH]^+$), 120 (100%, $[BtH]^+$) and 77 (45%, $[Ph]^+$); m/z (FAB, 3-NOBA) Found 238.93897 $C_{14}H_{12}N_3O$ requires 238.93951.

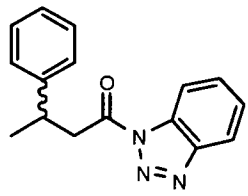
5.2.11 (*S*)-2-Methyl-butyryl benzotriazole (110)



The general protocol **5.2.8** was followed with benzotriazole (0.606g, 5.1mmols, 1 equiv.) dissolved in 'anhydrous' DCM (15ml). To this was added dropwise a solution of triethylamine (0.618g, 6.1mmols, 1.2 equiv.) and (*S*)-2-methyl-butyryl chloride **109** (0.675g, 5.6mmols, 1.1 equiv.) Column chromatography on silica gel eluting with DCM yielded the desired product as a clear oil (0.820g, 79%).

R_f (DCM) 0.48; υ_{max} (thin film)/cm^{-1} 2968, 2942, 2880 (CH), 1738 (C=O), 1606, 1595 (C=C), 1483, 1450 and 1387; δ_H ($CDCl_3$; 250MHz) 0.99 (3H, t, J 7.4Hz, $CH_2C\underline{H}_3$), 1.39 (3H, d, J 6.9Hz, $CHC\underline{H}_3$), 1.62-1.79 (1H, m, $C\underline{H}_aH_b$), 1.89-2.07 (1H, m, $CH_a\underline{H}_b$), 4.02 (1H, tq, J 6.9 and 6.9Hz, $C\underline{H}CH_3$), 7.48 (1H, ddd, J 8.2, 7.1 and 1.1Hz, $C\underline{H}_{bt}$), 7.63 (1H, ddd, J 8.2, 7.1 and 1.1Hz, $C\underline{H}_{bt}$), 8.10 (1H, ddd, J 8.2, 1.0 and 1.0Hz, $C\underline{H}_{bt}$) and 8.29 (1H, ddd, J 8.2, 1.1 and 1.0Hz, $C\underline{H}_{bt}$); δ_H ($CDCl_3$; 63MHz), 11.4 ($\underline{C}H_3$), 16.6 ($CH\underline{C}H_3$), 26.2 ($\underline{C}H_2$), 40.3 ($\underline{C}H$), 114.4 ($\underline{C}H_{bt}$), 119.9 ($\underline{C}H_{bt}$), 125.9 ($\underline{C}H_{bt}$), 130.2 ($\underline{C}H_{bt}$), 131.1 ($\underline{C}_{bt}$), 146.1 ($\underline{C}_{bt}$) and 176.2 (C=O); m/z (ESi +ve, 45kV) 204.4 (50%, $[MH]^+$); m/z (FAB, THIOG) 204 (70%, $[MH]^+$), 120 (100%, $[BtH]^+$); m/z (FAB, THIOG) Found 204.11373 $C_{11}H_{14}N_3O$ requires 204.11366; $[\alpha]_D^{20}$ -28.9 (0.994, $CHCl_3$).

5.2.12 (*R/S*)-1-Benzotriazol-1-yl-3-phenyl-butan-1-one (107)

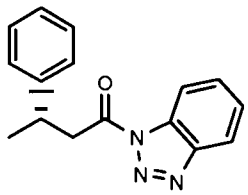


The general protocol **5.2.8** was followed with benzotriazole (3.0g, 25.2mmols, 1 equiv.) dissolved in 'anhydrous' DCM (75ml). To this was added dropwise a solution of triethylamine (3.54ml, 30.2mmols, 1.2 equiv.) and (*R/S*)-3-phenyl-butyryl chloride **106** (5.05g, 27.7mmols, 1.1 equiv.) In addition to the normal work-up procedure the organic layer was washed with $NaHCO_3$ (1M, 20ml), separated, dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to give a white solid (6.05g, 91%).

Note: (*R/S*)-**107** was analysed by normal phase chiral chromatography eluting on a gradient from Hexane:IPA: TFA (98:2:0.01) to Hexane: IPA: TFA (90:10:0.01) over 20 minutes with a column temperature of 2°C.

R_f (DCM) 0.74; R_t (*S*) 13.4mins, (*R*) 14.2; υ_{max} (thin film)/cm^{-1} 3024 (CH_{ar}), 2968, 2942, 2880 (CH), 1738 (C=O), 1606, 1595 (C=C), 1483, 1450 and 1387; δ_H ($CDCl_3$; 250MHz) 1.44 (3H, d, J 6.6Hz, $C\underline{H}_3$), 3.59-3.70 (2H, m, $C\underline{H}_2$), 3.76-3.86 (1H, m, C<u>H</u>), 7.15-7.36 (5H, m, Ph), 7.46 (1H, ddd, J 8.2, 7.1 and 1.1Hz, $C\underline{H}_{bt}$), 7.60 (1H, ddd, J 8.2, 7.1 and 1.1Hz, $C\underline{H}_{bt}$), 8.08 (1H, ddd, J 8.3, 1.0 and 1.0Hz, $C\underline{H}_{bt}$) and 8.23 (1H, ddd, J 8.3, 1.1 and 1.0Hz, $C\underline{H}_{bt}$); δ_H ($CDCl_3$; 63MHz), 21.9 ($\underline{C}H_3$), 35.9 (<u>C</u>H), 43.4 ($\underline{C}H_2$), 114.2 ($\underline{C}H_{bt}$), 119.9 ($\underline{C}H_{bt}$), 125.9 ($\underline{C}H_{bt}$), 126.6 ($\underline{C}H_{ar}$), 126.7 ($\underline{C}H_{ar}$ x 2), 128.4 ($\underline{C}H_{ar}$ x 2), 130.1 ($\underline{C}H_{bt}$), 130.8 ($\underline{C}_{bt}$), 145.9 ($\underline{C}_{bt}$), 146.6 ($\underline{C}_{ar}$) and 170.9 (C=O); m/z (ESi +ve, 45kV) 266.1 (100%, $[MH]^+$), 120 (50, $[BtH]^+$; m/z (FAB, 3-NOBA) 266 (80%, $[MH]^+$), 120 (90%, $[BtH]^+$) and 77 (35%, $[Ph]^+$); m/z (FAB, 3-NOBA) Found 265.12159 $C_{10}H_{12}N_3O$ requires 265.12151.

5.2.13 (*S*)-1-Benzotriazol-1-yl-3-phenyl-butan-1-one (107)



The general protocol (**5.2.8**) was followed with benzotriazole (0.30g, 2.52mmols, 1 equiv.) dissolved in 'anhydrous' DCM (10ml). To this was added dropwise a solution of triethylamine (0.360g, 3.0mmols, 1.2 equiv.) and (*S*)-3-phenylbutyryl chloride **106** (5.06g, 2.8mmols, 1.1 equiv.) Purification by column chromatography eluting with DCM provided the desired product as a colourless glass/oil (0.517g, 77%).

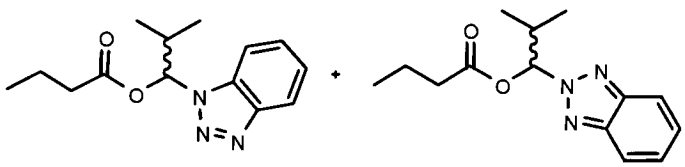
Note: The enantiomeric purity of (*S*)-**107** was confirmed by normal phase chiral chromatography eluting on a gradient from Hexane:IPA:TFA (98:2:0.01) to Hexane:IPA:TFA (90:10:0.01) over 20 minutes with a column temperature of 2°C. Analysis comparable to **5.2.13** (*R/S*)-**107** except; R_t (*S*) 13.4mins, (*R*) 14.2mins; m/z (FAB, 3-NOBA) Found 265.12139 $C_{16}H_{16}N_3O$ requires 265.12151; $[\alpha]_D^{20}$ – 10.6 (1.27, $CHCl_3$).

5.2.14 General procedure for preparation of benzotriazole esters

The *N*-acyl benzotriazole (1.0equiv.) was dissolved in the specified volume of acetonitrile. To this solution was added crushed potassium carbonate (10-25 mol %) and the appropriate aldehyde (1.05-1.1equiv.). The reaction mixture was allowed to stir at room temperature until TLC analysis indicated complete consumption of the *N*-acyl benzotriazole. The potassium carbonate was removed by filtration and the solvent removed under reduced pressure to provide a crude mixture containing the 1' and 2' esters. Column chromatography on silica gel eluting with the specified solvent system

provided the desired 'ester' product. Chiral integrity (where applicable) of the 'ester' products was determined by normal phase chromatography and the HPLC conditions are detailed along with the experimental details for each compound.

5.2.15 Butyric acid benzotriazol-1-ylmethyl ester (94a) and butyric acid benzotriazol-2-ylmethyl ester (95a)



The general protocol **5.2.14** was followed with **92** (1g, 5.29mmols) dissolved in acetonitrile (18ml). To this was added formaldehyde (37%, 0.415ml, 5.6mmols) and potassium carbonate (0.100g, 15mol%) and the resulting mixture stirred at room temperature for 16hours after which time the reaction mixture had turned cloudy. The reaction was worked up as normal. Column chromatography on silica gel eluting with DCM provided the ester products **94a** and **95a** as thick colourless oils (0.79g, 68%) and (0.04g, 3%).

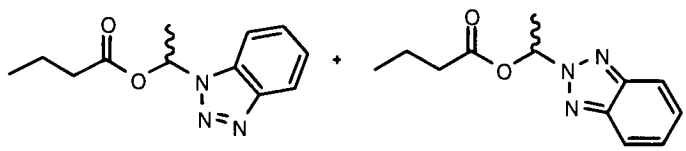
94a

R_f (DCM) 0.01; υ_{max} (thin film)/cm^{-1} 3054 (CH_{ar}), 2986, 2936, 2876 (CH), 1751 (C=O), 1616, 1591 (C=C), 1455, 1422; δ_H ($CDCl_3$; 250MHz) 0.86 (3H, t, J 7.4Hz, $C\underline{H}_3$), 1.61 (2H, tq, J 7.4 and 7.4Hz, $CH_3C\underline{H}_2CH_2$), 2.31 (1H, t, J 7.4Hz, $C\underline{H}_2C{=}O$), 6.58 (2H, s, $C\underline{H}_2O$), 7.40 (1H, ddd, J 8.3, 7.1 and 1.0Hz, $C\underline{H}_{bt}$), 7.54 (1H, ddd, J 8.3, 7.1 and 1.0Hz, $C\underline{H}_{bt}$), 7.76 (1H, ddd, J 8.3, 1.0 and 1.0Hz, $C\underline{H}_{bt}$) and 8.06 (1H, ddd, J 8.3, 1.0 and 1.0Hz, $C\underline{H}_{bt}$); δ_c ($CDCl_3$; 63MHz) 13.8 ($\underline{C}H_3$), 17.9 ($CH_3\underline{C}H_2$), 35.4 ($\underline{C}H_2C{=}O$), 67.5 ($\underline{C}H_2O$), 109.9 ($\underline{C}H_{bt}$), 119.9 ($\underline{C}H_{bt}$), 124.4 ($\underline{C}H_{bt}$), 128.2 ($\underline{C}H_{bt}$), 132.6 ($\underline{C}_{bt}$), 145.9 ($\underline{C}_{bt}$) and 172.6 (C=O); m/z (ESi +ve, 45kV) 220.2 (100%, $[MH^+]$); m/z (FAB, THIOG) 220 (95%, $[MH]^+$) and 120 (100%, $[BtH]^+$); m/z (FAB, THIOG) Found 220.10872 $C_{11}H_{14}N_3O_2$ requires 220.10858.

95a

R_f (DCM) 0.21; υ_{max} (thin film)/cm^{-1} 3061 (CH_{ar}), 2967, 2936, 2876 (CH), 1761 (C=O), 1563 (C=C), 1455; δ_H ($CDCl_3$; 250MHz) 0.91(3H, t, J 7.4Hz, $C\underline{H}_3$), 1.65 (2H, tq, J 7.4 and 7.4Hz, $CH_3C\underline{H}_2CH_2$), 2.37 (1H, t, J 7.4Hz, $C\underline{H}_2C{=}O$), 6.57 (2H, s, $C\underline{H}_2O$), 7.36-7.44 (2H, m, $C\underline{H}_{bt}$ x 2) and 7.84-7.91 (2H, m, $C\underline{H}_{bt}$ x 2); δ_c ($CDCl_3$; 63MHz) 13.4 ($\underline{C}H_3$), 17.9 ($CH_3\underline{C}H_2$), 35.5 ($\underline{C}H_2C{=}O$), 75.0 ($\underline{C}H_2O$), 118.5 ($\underline{C}H_{bt}$ x 2), 127.3 ($\underline{C}H_{bt}$ x 2), 144.8 ($\underline{C}_{bt}$ x 2) and 171.7 (C=O); m/z (ESi +ve, 45kV) 220.2 (55%, $[MH]^+$); m/z (FAB, THIOG) 220 (100%, $[MH]^+$) and 120 (75%, $[BtH]^+$); m/z (FAB, THIOG) Found 220.10864 $C_{11}H_{14}N_3O_2$ requires 220.10858.

5.2.16 Butyric acid-1-benzotriazol-1-ylethyl ester (94b) and butyric acid 1-benzotriazol-2-ylethyl ester (95b)



The general protocol **5.2.14** was followed with **92** (1g, 5.3mmols) dissolved in acetonitrile (15ml). To this was added acetaldehyde (0.277g, 5.6mmols) and potassium carbonate (0.182g, 25mol%) and the resulting mixture stirred at room temperature for 16 hours after which time the reaction mixture had turned cloudy. The reaction was worked up as normal and the crude product pre-absorbed on silica gel. Column chromatography on silica gel eluting with EtOAc:Hexane (4:1) provided the ester products **94b** and **95b** as thick colourless oils (0.92g, 75%) and (0.20g, 16%), respectively.

94b

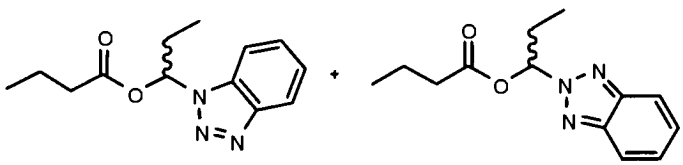
R_f (EtOAc:Hexane, 4:1) 0.3; υ_{max}(thin film)/cm^{-1} 3025 (CH_{ar}), 2965, 2937, 2875 (CH), 1746 (C=O), 1612, 1591, 1490 (C=C), 1453, 1413; δ_H ($CDCl_3$; 250MHz) 0.83 (3H, t, J 7.4Hz, $C\underline{H}_3$), 1.57 (2H, tq, J 7.4 and 7.4 Hz, $CH_3C\underline{H}_2CH_2$), 2.08 (3H, d, J 6.4Hz, CH_3), 2.25 (1H, dt, J 16.0 and 7.4Hz, $CH_2C\underline{H}_aH_bC{=}O$), 2.31 (1H, dt, J 16.0 and 7.7Hz, $CH_2CH_a\underline{H}_bC{=}O$), 7.37 (1H, ddd, J 8.3, 7.2 and 1.0Hz, $C\underline{H}_{bt}$) partially overlapping 7.40

(1H, q, J 6.4Hz, C<u>H</u>CH$_3$), 7.50 (1H, ddd, J 8.3, 7.2 and 1.0Hz, C<u>H</u>$_{bt}$), 7.73 (1H, ddd, J 8.3, 1.0 and 1.0Hz, C<u>H</u>$_{bt}$) and 8.04 (1H, ddd, J 8.3, 1.0 and 1.0Hz, C<u>H</u>$_{bt}$); δ_c ($CDCl_3$; 63MHz) 13.3 ($CH_2\underline{C}H_3$), 17.9 ($\underline{C}H_2$), 19.3 ($CH\underline{C}H_3$), 35.6 ($\underline{C}H_2C{=}O$), 76.0 ($\underline{C}H$), 110.1 ($\underline{C}H_{bt}$), 119.9 ($\underline{C}H_{bt}$), 124.2 ($\underline{C}H_{bt}$), 127.8 ($\underline{C}H_{bt}$), 132.1 ($\underline{C}_{bt}$), 145.8 ($\underline{C}_{bt}$) and 172.1 (C=O); m/z (ESi +ve, 45kV) 273.2 (30%, $[MK]^+$), 255.7 (70%, $[MNa]^+$) 234.1 (15%, $[MH]^+$); m/z (FAB, NOBA) 234 (100%, $[MH]^+$), 190 (70%, $[MH\text{-}CH_3CH_2CH_2C{=}O]^+$ 120 (90%, $[BtH]^+$); m/z (FAB, 3-NOBA) Found 233.11657 $C_{12}H_{16}N_3O_2$ requires 233.11643

95b

R_f (EtOAc:Hexane, 4:1) 0.49; υ_{max}(thin film)/cm^{-1} 3024 (CH_{ar}), 2967, 2942, 2877 (CH), 1754 (C=O), 1612, 1591, 1490 (C=C), 1453, 1413; δ_H ($CDCl_3$; 250MHz) 0.83(3H, t, J 7.4Hz, C<u>H</u>$_3$), 1.57 (2H, tq, J 7.4 and 7.4 Hz, $CH_3C\underline{H}_2CH_2$), 2.08 (3H, d, J 6.4Hz, C<u>H</u>$_3$), 2.25 (1H, dt, J 16.0 and 7.4Hz, $CH_2C\underline{H}_aH_bC{=}O$), 2.31 (1H, dt, J 16.0 and 7.7Hz, $CH_2CH_a\underline{H}_bC{=}O$), 7.34-7.44 (3H, m, C<u>H</u>$_{bt}$ x 2 overlapping with C<u>H</u>CH$_3$) and 7.83-7.93 (2H, m, C<u>H</u>$_{bt}$ x 2); δ_c ($CDCl_3$; 63MHz) 13.6 ($CH_2\underline{C}H_3$), 17.9 ($\underline{C}H_2$), 20.3 ($CH\underline{C}H_3$), 34.9 ($\underline{C}H_2C{=}O$), 84.1 ($\underline{C}H$), 117.6 ($\underline{C}H_{bt}$ x 2), 126.0 ($\underline{C}H_{bt}$ x 2), 143.3 ($\underline{C}_{bt}$ x 2) and 171.9 (C=O); m/z (ESi +ve, 45kV) 255.8 (25%, $[MNa]^+$) 233.7 (45%, $[MH]^+$); m/z (FAB, NOBA) 234 (30%, $[MH]^+$), 190 (50%, $[MH\text{-}CH_3CH_2CH_2C{=}O]^+$), 120 (100%, $[BtH]^+$); m/z (FAB, THIOG) Found 233.11650 $C_{13}H_{18}N_3O_2$ requires 233.11643.

5.2.17 Butyric acid-1-benzotriazol-1-ylpropyl ester (94c) and butyric acid 1-benzotriazol-2-ylpropyl ester (95c)



The general protocol **5.2.14** was followed with **92** (1g, 0.00529) dissolved in acetonitrile (15ml). To this was added propionaldehyde (0.322g, 0.0056mols) and potassium carbonate (0.182g, 25mol%) and the resulting mixture stirred at room

temperature for 16hours after which time the reaction mixture had turned cloudy. The reaction was worked up as normal and the crude product pre-absorbed on silica gel. Column chromatography on silica gel eluting with EtOAc:Hexane (4:1) provided the ester products **94c** and **95c** as thick colourless oils (0.89g, 68%) and (0.20g, 15%), respectively.

94c

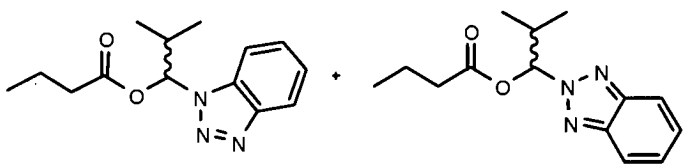
R_f (EtOAc: Hexane, 4:1) 0.29; υ_{max}(thin film)/cm^{-1} 3024 (CH_{ar}), 2967, 2942, 2877 (CH), 1746 (C=O), 1612, 1591, 1490 (C=C), 1453, 1413; δ_H ($CDCl_3$; 250MHz) 0.89(3H, t, J 7.4Hz, $C\underline{H}_3$), 0.99 (3H, t, J 7.4Hz, $C\underline{H}_3$), 1.63 (2H, tq, J 7.4 and 7.3Hz, $CH_3C\underline{H}_2CH_2$), 2.32 (1H, ddq, J 15.9, 7.4 and 7.3Hz, $CHC\underline{H}_aH_bCH_3$), 2.37 (1H, ddq, J 15.9, 7.4 and 7.3Hz, $CHCH_a\underline{H}_bCH_3$), 2.52 (1H, dt, J 14.4 and 7.3Hz, $CH_2C\underline{H}_aH_bC{=}O$) 2.58 (1H, dt, J 14.4 and 7.5Hz, $CH_2CH_a\underline{H}_bC{=}O$), 7.21 (1H, t, J 7.2Hz, $C\underline{H}CH_2CH_3$), 7.42 (1H, ddd, J 8.3, 7.2 and 1.0Hz, $C\underline{H}_{bt}$), 7.55 (1H, ddd, J 8.3, 7.2 and 1.0Hz, $C\underline{H}_{bt}$), 7.78 (1H, ddd, J 8.4, 1.0 and 1.0Hz, $C\underline{H}_{bt}$) and 8.10 (1H, ddd, J 8.4, 1.0 and 1.0Hz, $C\underline{H}_{bt}$); δ_c ($CDCl_3$; 63MHz) 9.6 ($\underline{C}H_3$), 13.8 ($\underline{C}H_3$), 18.5 ($\underline{C}H_2$), 26.9 ($\underline{C}H_2$), 36.2 ($\underline{C}H_2C{=}O$), 81.0 ($\underline{C}H$), 110.7 ($\underline{C}H_{bt}$), 120.5 ($\underline{C}H_{bt}$), 124.8 ($\underline{C}H_{bt}$), 128.4 ($\underline{C}H_{bt}$), 133.0 ($\underline{C}_{bt}$), 146.3 ($\underline{C}_{bt}$) and 172.8 (C=O); m/z (ESi +ve, 45kV) 285.8 (10%, $[MK]^+$), 269.9 (50%, $[MNa]^+$) 247.9 (5%, $[MH]^+$); m/z (FAB, NOBA) 248 (100%, $[MH]^+$), 190 (70%, $[MH\text{-}CH_3CH_2CH_2C{=}O]^+$ 120 (100%, $[BtH]^+$); m/z (FAB, 3-NOBA) Found 248.13967 $C_{13}H_{18}N_3O_2$ requires 248.13990.

95c

R_f (EtOAc:Hexane, 4:1) 0.46; υ_{max}(thin film)/cm^{-1} 3024 (CH_{ar}), 2967, 2942, 2877 (CH), 1752 (C=O), 1612, 1591, 1490 (C=C), 1453, 1413; δ_H ($CDCl_3$; 250MHz) 0.89 (3H, t, J 7.4Hz, $C\underline{H}_3$), 0.99 (3H, t, J 7.4Hz, $C\underline{H}_3$), 1.63 (2H, tq, J 7.4 and 7.3Hz, $CH_3C\underline{H}_2CH_2$), 2.32 (1H, ddq, J 15.9, 7.4 and 7.3Hz, $CHC\underline{H}_aH_bCH_3$), 2.37 (1H, ddq, J 15.9, 7.4 and 7.3Hz, $CHCH_a\underline{H}_bCH_3$), 2.52 (1H, dt, J 14.4 and 7.3Hz, $CH_2C\underline{H}_aH_bC{=}O$) 2.58 (1H, dt, J 14.4 and 7.5Hz, $CH_2CH_a\underline{H}_bC{=}O$), 7.18 (1H, t, J 7.2Hz, $C\underline{H}CH_2CH_3$), 7.34-7.44 (2H, m, $C\underline{H}_{bt}$ x 2) and 7.83-7.93 (2H, m, $C\underline{H}_{bt}$ x 2); δ_c ($CDCl_3$; 63MHz) 7.8 ($\underline{C}H_3$), 12.6 ($\underline{C}H_3$),

17.3 ($\underline{C}H_2$), 26.5 ($\underline{C}H_2$), 34.9 ($\underline{C}H_2C=O$), 85.9 ($\underline{C}H$), 117.8 ($\underline{C}H_{bt}$ x 2), 126.2 ($\underline{C}H_{bt}$ x 2), 143.4 ($\underline{C}_{bt}$ x 2) and 170.9 (C=O); m/z (ESi +ve, 45kV) 285.8 (10%, $[MK]^+$), 269.9 (50%, $[MNa]^+$) 247.9 (5%, $[MH]^+$); m/z (FAB, NOBA) 248 (30%, $[MH]^+$), 190 (50%, $[MH-CH_3CH_2CH_2C=O]^+$), 120 (100%, $[BtH]^+$); m/z (FAB, THIOG) Found 248.13951 $C_{13}H_{18}N_3O_2$ requires 248.13990.

5.2.18 Butyric acid-1-benzotriazol-1-yl-2-methyl-propyl ester (94d) and butyric acid-1-benzotriazol-2-yl-2-methyl-propyl ester (95d)



The general protocol **5.2.14** was followed with **92** (1g, 5.3mmols) dissolved in acetonitrile (12ml). To this was added *iso*-butyraldehyde (0.485ml, 5.6mmols) and potassium carbonate (0.16g, 25mol%) and the resulting mixture stirred at room temperature for 16 hours. Column chromatography on silica gel eluting with DCM provided the title compounds **94d** and **95d** as thick colourless oils (1g, 72%) and (0.215g, 16%), respectively.

94d

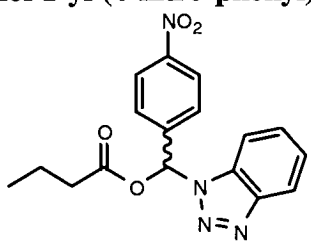
R_f (DCM) 0.1; R_t 9.8 and 10.6 mins; υ_{max} (thin film)/cm^{-1} 3063 (CH_{ar}), 2967, 2935, 2876 (CH), 1750 (C=O), 1612, 1490 (C=C), 1452; δ_H ($CDCl_3$; 250MHz) 0.79 (3H, d, J 6.8Hz, $CHC\underline{H}_3$), 0.88 (3H, t, J 7.4Hz, $C\underline{H}_3CH_2$), 1.22 (3H, d, J 6.7Hz, $CHC\underline{H}_3$), 1.55-1.7 (2H, m $CH_3C\underline{H}_2$), 2.25-2.45 (2H, m, $C\underline{H}_2C=O$), 2.95-3.10 (1H, dqq, J 9.7, 6.7 and 6.7Hz, $C\underline{H}(CH_3)_2$), 6.91 (1H, d, J 9.7Hz,$C\underline{H}CH(CH_3)_2$), 7.41 (1H, ddd, J 8.4, 7.0 and 1.0Hz, $C\underline{H}_{bt}$), 7.54 (1H, ddd, J 8.4, 7.0 and 1.0Hz, $C\underline{H}_{bt}$), 7.78 (1H, ddd, J 8.4, 1.0 and 0.9Hz, $C\underline{H}_{bt}$) and 8.09 (1H, ddd, J 8.4, 1.0 and 0.9Hz, $C\underline{H}_{bt}$); δ_H ($CDCl_3$; 63MHz) 13.9 ($\underline{C}H_3$), 18.2 ($CH\underline{C}H_3$), 18.5 ($CH_3\underline{C}H_2$), 18.9 ($CH\underline{C}H_3$) 33.3 ($\underline{C}H(CH_3)_2$), 36.2

($\underline{C}H_2C=O$), 84.4 ($\underline{C}HCH(CH_3)_2$), 110.7 ($\underline{C}H_{bt}$), 120.4 ($\underline{C}H_{bt}$), 124.7 ($\underline{C}H_{bt}$), 128.4 ($\underline{C}H_{bt}$), 133.2 ($\underline{C}_{bt}$), 146.2 ($\underline{C}_{bt}$) and 172.8 (C=O); m/z (ESi +ve, 45kV) 262.3 (70%, $[MH]^+$); m/z (FAB, NOBA) 262 (35%, $[MH]^+$),120 (100%, $[BtH]^+$); m/z (FAB, THIOG) Found 262.15521 $C_{14}H_{20}N_3O_2$ requires 262.15553.

95d

R_f (DCM) 0.3; R_t 6.5 and 10.1 mins; υ_{max}(thin film)/cm^{-1} 3063 (CH_{ar}), 2967, 2935, 2876 (CH), 1755 (C=O), 1612, 1562 (C=C), 1466 (C=C), 1451; δ_H ($CDCl_3$; 250MHz) 0.80 (3H, d, J 6.8Hz, $CHC\underline{H}_3$), 0.93 (3H, t, J 7.4Hz, $C\underline{H}_3CH_2$), 1.16 (3H, d, J 6.8Hz, $CHC\underline{H}_3$), 1.55-1.7 (2H, m, $CH_3C\underline{H}_2$), 2.25-2.45 (2H, m, $CH_2C=O$), 2.95-3.10 (1H, dqq, J 9.7, 6.7 and 6.7Hz, $C\underline{H}(CH_3)_2$), 6.96 (1H, d, J 9.0Hz,$C\underline{H}CH(CH_3)_2$), 7.39-7.44 (2H, m, $C\underline{H}_{bt}$ x 2) and 7.89-7.94 (2H, m, $C\underline{H}_{bt}$); δ_H ($CDCl_3$; 63MHz) 13.9 ($\underline{C}H_3$), 17.8 ($CH\underline{C}H_3$),18.3 ($CH\underline{C}H_3$) 18.5 ($CH_3\underline{C}H_2$), 32.2 ($\underline{C}H(CH_3)_2$), 36.1 ($\underline{C}H_2C=O$), 90.1 ($\underline{C}HCH(CH_3)_2$), 119.1 ($\underline{C}H_{bt}$ x 2), 127.4 ($\underline{C}H_{bt}$ x 2), 144.5 ($\underline{C}_{bt}$ x 2) and 172.8 (C=O) 84.4 ($\underline{C}HCH(CH_3)_2$), 110.7 ($\underline{C}H_{bt}$), 120.4 ($\underline{C}H_{bt}$), 124.7 ($\underline{C}H_{bt}$), 128.4 ($\underline{C}H_{bt}$), 133.2 ($\underline{C}_{bt}$), 146.2 ($\underline{C}_{bt}$) and 172.8 ($\underline{C}$=O); m/z (ESi +ve, 45kV) 284.5 (70%, $[MNa]^+$), 262.6 (25%, $[MH]^+$); m/z (FAB, NOBA) 262 (55%, $[MH]^+$), 120 (100%, $[BtH]^+$); m/z (FAB, 3-NOBA) Found 262.15579 $C_{14}H_{20}N_3O_2$ requires 262.15553.

5.2.19 Butyric acid benzotriazol-1-yl-(4-nitro-phenyl)-methyl ester - (94e)

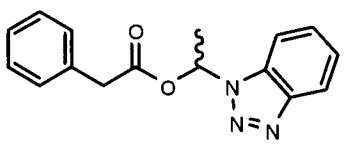


The general protocol (**5.2.14**) was followed with **92** (2g, 10.6mmols) dissolved in acetonitrile (24ml). To this was added *p*-nitrobenzaldehyde (1.38g, 11.1mmols) and potassium carbonate (0.3g, 25mol%) and the resulting mixture stirred at room temperature for 16 hours. The reaction went purple during stirring and was worked up as

normal. Column chromatography on silica gel eluting with EtOAc:Hexane (4:1) provided the 1-yl ester product as a thick colourless oil (2.15g, 62%). The 2' **(95e)** isomer could not be isolated in pure form due to contamination with *p*-nitrobenzaldehyde.

R_f (DCM) 0.21; R_t 10.8 and 14.7 mins; υ_{max}(thin film)/cm^{-1} 3063 (CH_{ar}), 2964, 2937, 2875 (CH), 1756 (C=O), 1608, 1526 (C-NO_2), 1492 (C=C), 1451 and 1348 (C-NO_2); δ_H ($CDCl_3$; 250MHz) 0.89 (3H, t, J 7.4Hz, $C\underline{H}_3$), 1.67 (2H, tq, J 7.4 and 7.4Hz, $CH_3C\underline{H}_2$), 2.44 (1H, dt, J 16.2 and 7.2Hz, $C\underline{H}_aH_bC{=}O$), 2.50 (1H, dt, 16.2 and 7.6Hz, $CH_a\underline{H}_bC{=}O$), 7.35-7.50 (3H, m, $C\underline{H}_{ar}$ x 3), 7.62 (1H, ddd, J 8.2, 7.0 and 1.0Hz, $C\underline{H}_{bt}$), 7.65 (1H, ddd, J 8.2, 7.0 and 1.0Hz, $C\underline{H}_{bt}$), 8.05 (1H, ddd, J 8.2, 1.0 and 1.0Hz, $C\underline{H}_{bt}$), 8.22-8.28 (2H, m, CH_{ar}+ CH_{bt} overlapping) and 8.50 (1H, s, $C\underline{H}OC{=}O$); δ_H ($CDCl_3$; 63MHz) 13.3 ($\underline{C}H_3$), 17.9 ($CH_3\underline{C}H_2$), 35.5 ($\underline{C}H_2C{=}O$), 78.5 ($\underline{C}HO$), 109.9 ($\underline{C}H_{bt}$), 120.3 ($\underline{C}H_{bt}$), 123.9 ($\underline{C}H_{ar}$ x 2), 124.6 ($\underline{C}H_{bt}$), 124.6 ($\underline{C}H_{bt}$), 127.4 ($\underline{C}H_{ar}$ x 2), 128.4 ($\underline{C}H_{bt}$), 131.7 ($\underline{C}_{bt}$), 140.7 ($\underline{C}_{ar}$), 146.0 ($\underline{C}_{bt}$), 148.4 ($\underline{C}_{ar}$) and 171.2 (C=O); m/z (ESi +ve, 45kV) 363.9 (20%, $[MNa]^+$) 341 (40%, $[MH]^+$); m/z (FAB, NOBA) 341 (50%, $[MH]^+$), 120 (100%, $[BtH]^+$); m/z (FAB, NOBA) Found 341.12442 $C_{17}H_{16}N_4O_4$ requires 341.12498.

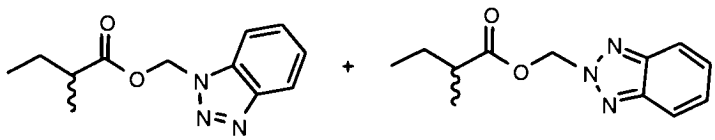
5.2.20 Phenyl-acetic acid 1-benzotriazol-1-yl-ethyl ester (96)



The general protocol **5.2.14** was followed with **93** (1g, 4.4mmols) dissolved in acetonitrile (12ml). To this was added acetaldehyde (0.215g, 4.8mmols, 1.1 equiv.) and potassium carbonate (0.15g, 25mol%) and the resulting mixture stirred at room temperature for 16hours. Column chromatography on silica gel eluting with EtOAc:Hexane (4:1) provided the title compound as a thick colourless oil (0.89g, 75%). The 2' isomer was not isolated.

R_f (EtOAc:Hexane, 4:1) 0.15; υ_{max}(thin film)/cm^{-1} 3052, 3024 (CH_{ar}), 2999, 2942 (CH), 1747 (C=O), 1611, 1492 (C=C), 1462, 1408; δ_H ($CDCl_3$; 250MHz) 2.09 (3H, d, J 6.3Hz, $C\underline{H}_3$), 3.59 (1H, d, J 15.4Hz, $C\underline{H}_aH_bC{=}O$), 3.63 (1H, d, J 15.4Hz, $CH_a\underline{H}_bC{=}O$), 7.14-7.27 (5H, m, $C\underline{H}_{ar}$ x 5), 7.33-7.40 (2H, m, $C\underline{H}_{bt}$ and $C\underline{H}CH_3$ overlapping), 7.46 (1H, ddd, J 8.2, 6.9 and 1.2Hz, $C\underline{H}_{bt}$), 7.61 (1H, ddd, J 8.2, 1.0 and 1.0Hz, $C\underline{H}_{bt}$) and 8.05 (1H, ddd, J 8.2, 1.0 and 1.0Hz, $C\underline{H}_{bt}$); δ_H ($CDCl_3$; 63MHz) 19.2 ($\underline{C}H_3$), 40.7 ($\underline{C}H_2C{=}O$), 77.4 ($\underline{C}H$), 110.1 ($\underline{C}H_{bt}$), 119.8 ($\underline{C}H_{bt}$), 124.2 ($\underline{C}H_{bt}$), 127.2 ($\underline{C}H_{bt}$), 127.8 ($\underline{C}H_{ar}$), 128.5 ($\underline{C}H_{ar}$ x 2), 129.0 ($\underline{C}H_{ar}$ x 2), 132.0 ($\underline{C}_{ar}$), 132.6 ($\underline{C}_{bt}$), 145.8 ($\underline{C}_{bt}$) and 170.1 (C=O); m/z (ESi +ve, 45kV) 319.6 (10%, $[MK]^+$), 303.7 (55%, $[MNa]^+$) 281.7 (65%, $[MH]^+$); m/z (FAB, NOBA) 282 (100%, $[MH]^+$), 146 (65%, $[MH\text{-}PhCH_2CO_2]^+$), 120 (100%, $[BtH]^+$), 91 (100%, $[PhCH_2]^+$) and 77 (50%, $[Ph]^+$); m/z (FAB, 3-NOBA) Found 281.12422 $C_{10}H_{12}N_3O$ requires 281.12425.

5.2.21 (*R/S*)-2-methyl butyric acid benzotriazol-1-yl methyl ester (111A) and 2-methyl butyric acid benzotriazole-2-yl-methyl ester (111B)



The general protocol **5.2.14** was followed with (*R/S*)-**110** (0.2g, 0.98mmols) dissolved in acetonitrile (3ml). To this was added formaldehyde (0.031g, 1.08mmols) and potassium carbonate (0.03g, 25mol%) and the resulting mixture stirred at room temperature for 16hours after which time the reaction mixture had turned cloudy. The reaction was worked up as normal and the crude product pre-absorbed on silica gel. Column chromatography on silica gel eluting with DCM provided the desired ester products (*R/S*)-**111A** and (*R/S*)-**111B** as thick colourless oils (0.31g, 67%) and (0.014, 3%), respectively. Ester (*R/S*)-**111A** was analysed by normal phase chiral HPLC eluting with hexane: IPA:TFA (95:5:0.1) with a column temperature of 5°C

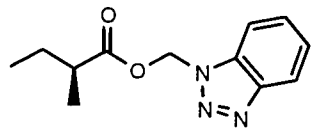
(*R/S*)-**111A**

R_f (DCM) 0.14; R_t (*S*) 12.9 minutes, (*R*) 14.1 minutes; υ_{max}(thin film)/cm^{-1} 3043 (CH_{ar}), 2971, 2937, 2878 (CH), 1748 (C=O), 1616, 1593 (C=C), 1495, 1455; δ_H ($CDCl_3$; 250MHz) 0.75 (3H, t, J 7.5Hz, C$\underline{H}_3$), 1.08 (3H, d, J 7.0Hz, CHC$\underline{H}_3$), 1.38-1.49 (1H, m, C$\underline{H}_a$H$_b$CH), 1.51-1.63 (1H, m, CH$_a\underline{H}_b$CH), 2.39 (1H, tq, J 7.0 and 7.0Hz, C$\underline{H}$CH$_3$), 6.58 (2H, s, C$\underline{H}_2$O), 7.39 (1H, ddd, J 8.3, 7.0 and 1.1Hz, C$\underline{H}_{bt}$), 7.54 (1H, ddd, J 8.3, 7.0 and 1.1Hz, C$\underline{H}_{bt}$), 7.74 (1H, ddd, J 8.3, 1.0 and 1.0Hz, C$\underline{H}_{bt}$) and 8.05 (1H, ddd, J 8.3, 1.0 and 1.0Hz, C$\underline{H}_{bt}$); δ_c ($CDCl_3$; 63MHz) 11.2 (C$\underline{H}_3$), 16.1 (CHC$\underline{H}_3$), 26.3 (C$\underline{H}_2$), 40.5 (C$\underline{H}$C=O), 67.6 (C$\underline{H}_2$O), 109.9 (C$\underline{H}_{bt}$), 119.9 (C$\underline{H}_{bt}$), 124.4 (C$\underline{H}_{bt}$), 128.3 (C$\underline{H}_{bt}$), 132.5 (C$\underline{}_{bt}$), 145.9 (C$\underline{}_{bt}$) and 175.6 (C=O); m/z (ESi +ve, 45kV) 234.2 (100, $[MH]^+$); m/z (FAB, THIOG) 234 (90%, $[MH]^+$), 120 (80%, $[BtH]^+$); m/z (FAB, THIOG) Found 234.12437 $C_{12}H_{16}N_3O_2$ requires 234.12423.

(*R/S*)-**111B**

R_f (DCM) 0.36; υ_{max}(thin film)/cm^{-1} 3054 (CH_{ar}), 2979, 2939, 2880 (CH), 1755 (C=O), 1616, 1593 (C=C), 1495, 1455; δ_H ($CDCl_3$; 250MHz) 0.78 (3H, t, J 7.3Hz, C$\underline{H}_3$), 1.10 (3H, d, J 7.0Hz, CHC$\underline{H}_3$), 1.38-1.50 (1H, m, C$\underline{H}_a$H$_b$CH), 1.49-1.62 (1H, m, CH$_a\underline{H}_b$CH), 2.39 (1H, tq, J 7.0 and 7.0Hz, C$\underline{H}$CH$_3$), 6.68 (2H, s, C$\underline{H}_2$O), 7.39- 7.44 (2H, m, 2 x C$\underline{H}_{bt}$), 7.85-7.95 (2H, m, C$\underline{H}_{bt}$); δ_c ($CDCl_3$; 63MHz) 11.2 (C$\underline{H}_3$), 16.2 (CHC$\underline{H}_3$), 26.4 (C$\underline{H}_2$), 40.5 (C$\underline{H}$C=O), 70.9 (C$\underline{H}_2$O), 119.6 (C$\underline{H}_{bt}$ x 2), 127.6 (C$\underline{H}_{bt}$ x 2), 143.2 (C$\underline{}_{bt}$ x 2) and 173.6 (C=O); m/z (ESi +ve, 45kV) 234.2 (100, $[MH]^+$); m/z (FAB, THIOG) 234 (90%, $[MH]^+$), 120 (80%, $[BtH]^+$); m/z (FAB, THIOG) Found 234.12440 $C_{12}H_{16}N_3O_2$ requires 234.12423.

5.2.22 (*S*)-2-methyl butyric acid benzotriazol-1-yl methyl ester (111A)

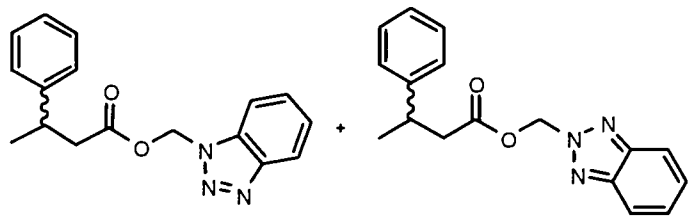


The general protocol **5.2.14** was followed with (*S*)-**110** (0.2g, 0.98mmols) dissolved in acetonitrile (3ml). To this was added formaldehyde (0.031g, 1.08mmols) and potassium carbonate (0.03g, 25mol%) and the resulting mixture stirred at room temperature for 16hours after which time the reaction mixture had turned cloudy. The reaction was worked up as normal and the crude product pre-absorbed on silica gel. Column chromatography on silica gel eluting with DCM provided the desired ester product as a thick colourless oil (0.110g, 47%). Ester (*S*)-**111A** was analysed by normal phase chiral HPLC eluting with hexane: IPA: TFA (95:5:0.1) with a column temperature of 5°C and determined to be a 87:13 mixture of enantiomers (74% e.e.).

(*S*)-**111A**

All analysis comparable to (*R/S*)–111A except m/z (FAB, THIOG) Found 234.12424 $C_{12}H_{16}N_3O_2$ requires 234.12423; $[\alpha]_D^{20}$ +13.4 (0.972, $CHCl_3$).

5.2.23 (*R/S*)-3-Phenylbutyric acid benzotriazol-1-ylmethyl ester (105) and (*R/S*) 3-phenylbutyric acid benzotriazol-2-ylmethyl ester (108).



The general protocol **5.2.14** was followed with (*R/S*)-**107** (1.13g, 4.26mmols) dissolved in acetonitrile (10ml). To this was added formaldehyde (0.132g, 4.47mmols, 1.05equivs) and potassium carbonate (0.088g, 15mol%) and the resulting mixture stirred at room temperature for 16hours. Column chromatography on silica gel eluting with DCM provided the ester products **105** and **108** as thick colourless oils (0.924g, 73%) and

(0.023g, 2%), respectively. Ester (*R/S*)-**105** was analysed by normal phase chiral HPLC eluting with hexane: IPA (70:30) with a column temperature of 5°c and determined to be a 50:50 mixture of enantiomers.

(*R/S*)-**105**

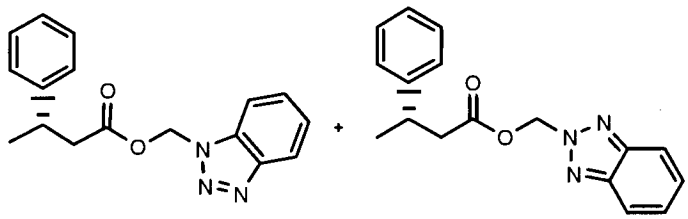
R_f (DCM) 0.27; R_t (*R*) 11.9 minutes, (*S*) 17.5 minutes; υ_{max}(thin film)/cm^{-1} 3062, 3028 (CH_{ar}), 2966, 2928, 2878 (CH), 1750 (C=O), 1616, 1593 (C=C), 1495, 1454; δ_H ($CDCl_3$; 250MHz) 1.21 (3H, d, J 7.0, $CHC\underline{H}_3$), 2.61 (1H, dd, J 15.2 and 7.7Hz, $C\underline{H}_aH_bCH$), 2.63 (1H, dd, J 15.2 and 7.3Hz, $CH_a\underline{H}_bCH$), 3.21 (1H, m, $C\underline{H}CH_3$), 6.48 (1H, d, J 11.2Hz, $C\underline{H}_aH_bO$), 6.51 (1H, d, J 11.2Hz, $CH_a\underline{H}_bO$), 7.00-7.15 (5H, m, Ar$\underline{H}$ x 5), 7.39 (1H, ddd, J 8.2, 6.9 and 1.2Hz, $C\underline{H}_{bt}$), 7.48 (1H, ddd, J 8.2, 6.9 and 1.2Hz, $C\underline{H}_{bt}$), 7.58 (1H, ddd, J 8.2, 1.2 and 1.1Hz, $C\underline{H}_{bt}$) and 8.05 (1H, ddd, J 8.3, 1.1 and 1.0Hz, $C\underline{H}_{bt}$); δ_c ($CDCl_3$; 63MHz) 21.7 ($\underline{C}H_3$), 36.2 ($\underline{C}H$), 42.2 ($\underline{C}H_2C=O$), 67.3 ($\underline{C}H_2O$), 109.9 ($\underline{C}H_{bt}$), 119.8 ($\underline{C}H_{bt}$), 124.3 ($\underline{C}H_{bt}$), 126.3 ($\underline{C}H_{ar}$), 126.3 ($\underline{C}H_{ar}$), 128.2 ($\underline{C}H_{bt}$), 128.2 ($\underline{C}H_{ar}$ x 2), 132.4 ($\underline{C}_{bt}$), 144.4 ($\underline{C}_{ar}$), 145.9 ($\underline{C}_{bt}$) and 171.2 (C=O); m/z (ESi +ve, 45kV) 318.1 (100%, $[MNa]^+$) 296.1 (30%, $[MH]^+$); m/z (FAB, 3-NOBA) 296 (50%, $[MH]^+$), 120 (100%, $[BtH]^+$) and 77 (25%, $[Ph]^+$); m/z (FAB, 3-NOBA) Found 296.13972 $C_{17}H_{18}N_3O_2$ requires 296.13990.

(*R/S*)-**108**

R_f (DCM) 0.61; υ_{max}(thin film)/cm^{-1} 3043 (CH_{ar}), 2971, 2937, 2878 (CH), 1754 (C=O), 1593 (C=C), 1495, 1455; δ_H ($CDCl_3$; 250MHz) 1.21 (3H, d, J 7.0, $CHC\underline{H}_3$), 2.66 (1H, dd, J 15.5 and 7.9Hz, $C\underline{H}_aH_bCH$), 2.63 (1H, dd, J 15.5 and 7.3Hz, $CH_a\underline{H}_bCH$), 3.21 (1H, m, $C\underline{H}CH_3$), 6.48 (1H, d, J 9.9Hz, $C\underline{H}_aH_bO$), 6.51 (1H, d, J 9.9Hz, $CH_a\underline{H}_bO$), 7.00-7.29 (5H, m, Ar$\underline{H}$ x 5) 7.38–7.46 (2H, m, $C\underline{H}_{bt}$ x 2) and 7.84-7.91 (2H, m, $C\underline{H}_{bt}$ x 2); δ_c ($CDCl_3$; 63MHz) 21.6 ($\underline{C}H_3$), 36.1 ($\underline{C}H$), 42.2 (CH_2), 74.9 ($\underline{C}H_2O$), 118.5 ($\underline{C}H_{bt}$ x 2), 126.5 ($\underline{C}H_{ar}$ x 2), 127.3 ($\underline{C}H_{ar}$ x 2), 128.4 ($\underline{C}H_{ar}$ x 2), 144.8 ($\underline{C}_{ar}$), 144.9 ($\underline{C}_{bt}$ x 2) and 170.4 (C=O); m/z (ESi +ve, 45kV) 318.1 (15%, $[MNa]^+$) 296.1 (30%, $[MH]^+$); m/z

(FAB, 3-NOBA) 296 (60%, $[MH]^+$), 120 (100%, $[BtH]^+$) and 77 (25%, $[Ph]^+$); m/z (FAB, 3-NOBA) Found 296.13984 $C_{17}H_{18}N_3O_2$ requires 296.13990.

5.2.24 (*S*)-3-phenylbutyric acid benzotriazol-1-ylmethyl ester (105) and (*S*)-3-phenylbutyric acid benzotriazol-2-ylmethyl ester (108)



The general protocol **5.2.14** was followed with (*S*)-**107** (0.569g, 2.14mmols) dissolved in acetonitrile (5ml). To this was added formaldehyde (0.183g, 2.25mmols, 1.05equivs) and potassium carbonate (0.045g, 15mol %) and the resulting mixture stirred at room temperature for 16hours. Column chromatography on silica gel eluting with DCM provided the ester products (*S*)-**105** and (*S*)-**108** as thick colourless oils (0.443g, 70%) and (0.010g, 1.6%). Ester (*S*)-**105** was analysed by normal phase chiral HPLC eluting with hexane: IPA (70:30) with a column temperature of 5°C and determined to be a 99.6:0.4 mixture of enantiomers. (>99% e.e.)

(*S*)-**105**

All analysis comparable to **5.2.23** except m/z (FAB, 3-NOBA) Found 296.13972 $C_{17}H_{18}N_3O_2$ requires 296.13990; $[\alpha]_D^{20}$ +29.4 (1.0, $CHCl_3$).

(*S*)-**108**

All analysis comparable to **5.2.23** except m/z (FAB, 3-NOBA) Found 296.13984 $C_{17}H_{18}N_3O_2$ requires 296.13990.

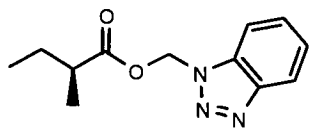
5.2.25 Alternative methods of preparing benzotriazol-1yl-esters from α–chiral acids

5.2.26 General protocol for coupling 1-Hydroxymethylbenzotriazole with 2-methylbutyric acid.

1-Hydroxymethylbenzotriazole **100** (1equiv.) and (*R/S*)-2-methyl butyric acid (1equiv.) was dissolved in 'anhydrous' DCM. The appropriate coupling reagents (1equiv.) and base (1 equiv.) were added. The reactions were allowed to stir overnight at room temperature then analysed by gradient reverse phase HPLC eluting from 5:95 (CH_3CN:H_2O, 0.1%TFA) to 95:5 (CH_3CN:H_2O, 0.1%TFA) over 30mins.

Component	R_t (mins)
Benzotriazole	13.4
100	13.4
1' ester (*R/S*)-**111**	20.6
2' ester (*R/S*)-**112**	21.8
N-acyl Bt (*R/S*)-**110**	23.1

5.2.27 (*S*)-2-Methyl butyric acid benzotriazol-1-ylmethyl ester (111A) via Mitsunobu reaction



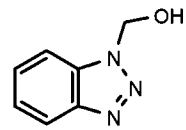
Triphenylphosphine (1.31g, 5mmols, 1equiv.) and 1-hydroxymethyl benzotriazole, **100** (0.82g, 5.5mmols, 1.1equiv.) were dissolved in 'anhydrous' THF (5ml). This solution was added dropwise to a solution of DEAD (0.87g, 5mmols, 1equiv.) and (*S*) 2-methyl butyric acid **102** (0.56g, 5mmols, 1equiv.) in 'anhydrous' THF (5ml). The reaction was allowed to stir overnight at room temperature after which time the solution had decolourised. The solvent was removed under reduced pressure and the crude mixture suspended in ether. The white precipitate formed was removed by

filtration. The crude material was further purified by column chromatography on silica gel eluting with DCM: EtOAc (4:1) to provide the title compound as a colourless oil (0.91g, 78%).

The same procedure was used for entries 5 and 6 (Table 5) except 'anhydrous' DMF or resin bound PPh_3 were used, respectively.

Analysis comparable to **5.2.22** except; Ester (*S*)-**111** was analysed by normal phase chiral HPLC eluting with hexane: IPA:TFA (95:5:0.1) with a column temperature of 5°C and determined to be a 99:1 mixture of enantiomers (>98% e.e. – (*S*)). m/z (FAB, THIOG) Found 234.12437 $C_{12}H_{16}N_3O_2$ requires 234.12423; $[\alpha]_D^{20}$ +17.9 (0.98, $CHCl_3$).

5.2.28 1-Hydroxymethyl benzotriazole 100



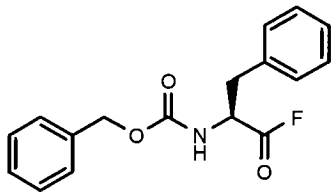
100

Benzotriazole (6g, 0.05mols) and formaldehyde (37%, 4.32ml) were dissolved in a mixture of glacial acetic acid and H_2O (15ml, 1:2) and stirred overnight at room temperature. A white precipitate formed which was isolated by filtration and dried to constant weight under reduced pressure over P_2O_5 to provide the desired product as a white powder (7.01g, 93%).

R_f (DCM: Hexane) 0.28, R_f Bt = 0.28; R_f (DCM) 0.47, R_f Bt = 0.47; Mp 149-150°C, Lit.[139] Mp.148-149°C; υ_{max} (nujol)/cm^{-1} 3171 (OH), 1614 (C=C), 1591 (C=C), 1494; δ_H ($(CD_3)_2SO$; 250MHz) 6.04 (2H, d, J 7.3Hz, C<u>H</u>$_2$O), 7.24 (1H, t, J 7.4Hz, CH$_2$O<u>H</u>), 7.42 (1H, ddd, J 8.0, 7.0 and 0.9Hz, C<u>H</u>$_{bt}$), 7.57 (1H, ddd, J 8.0, 7.0 and 0.9Hz, C<u>H</u>$_{bt}$), 7.91 (1H, br d, J 8.0Hz, C<u>H</u>$_{bt}$) and 8.06 (1H, br d, J 8.0Hz, C<u>H</u>$_{bt}$); δ_H ($(CD_3)_2SO$; 63MHz) 70.4 (<u>C</u>H$_2$O), 111.1 (<u>C</u>H$_{bt}$), 119.2 (<u>C</u>H$_{bt}$), 124.2 (<u>C</u>H$_{bt}$), 127.5 (<u>C</u>H$_{bt}$), 132.4 (<u>C</u>$_{bt}$) and 145.7 (<u>C</u>$_{bt}$); m/z (ESi, +ve) 120 (100%, $[MH\text{-}CH_2OH]^+$); m/z (FAB, NOBA) 120 (10%, $[MH\text{-}CH_2OH]^+$).

5.2.29 Preparation of protected amino acid containing substrates

5.2.30 Carboxybenzyloxy–*L*-phenylalanine fluoride (113)

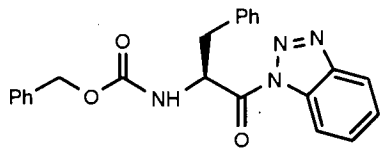


Carboxybenzyloxy-*L*-phenylalanine (3g, 0.01mols, 1 equiv.) and pyridine (1 equiv., 0.808ml, 0.01mols) were suspended in 'anhydrous' DCM (20ml) under an inert atmosphere of nitrogen. The reaction mixture was cooled to –15°C in an salt/ice bath and cyanuric fluoride (1 equiv., 1.35g, 0.01mols) dissolved in 'anhydrous' DCM (10ml) was added dropwise. A white precipitate formed on addition of the cyanuric fluoride and the reaction mixture was stirred at –15°C for 2 hours. After this time crushed ice and further DCM (20ml) were added and the organic layer separated. The aqueous layer was re-extracted with further DCM (10ml). The combined organic layers were washed with ice-cold H_2O, separated, dried over $MgSO_4$, filtered and the solvent removed to provide the desired product as an off-white solid. The product was purified by recrystallisation from EtOAc to provide the product as a white powder (2.3g, 77%).

Mp 81-82°C, Lit[143] 80-83°C; υ_{max}(nujol)/cm^{-1} 3324 (NH), 3029 (CH_{ar}), 1832 (C=O, acid fluoride), 1676 (C=O, urethane), 1535 (C=O, urethane); δ_H ($CDCl_3$; 250MHz) 3.17 (2H, br d, J 5.5Hz, CHC<u>H</u>$_2$), 4.8-4.86 (1H, m, C<u>H</u>CH$_2$), 5.11 (2H, s, CH_2OCO) 5.15 (1H, obscured by CH_2OCO, N<u>H</u>), 7.13 – 7.26 (2H, m, C<u>H</u>$_{ar}$ x 2) and 7.28-7.41 (8H, m, C<u>H</u>$_{ar}$); δ_C ($CDCl_3$; 63MHz) 36.7 (<u>C</u>H$_2$), 53.1 and 54.1 (<u>C</u>H, split by COF, J 60Hz), 67.3 (<u>C</u>H$_2$OCO), 127.7 (<u>C</u>H$_{ar}$), 128.1 (<u>C</u>H$_{ar}$ x 2) 128.3 (<u>C</u>H$_{ar}$), 128.5 (<u>C</u>H$_{ar}$ x 2), 128.9 (<u>C</u>H$_{ar}$ x 2), 129.0 (<u>C</u>H$_{ar}$ x 2), 134.0 (<u>C</u>$_{ar}$), 135.6 (<u>C</u>$_{ar}$), 155.4 (OC=O), 158.7 and 164.6 (COF, split by ^{19}F, J 370Hz); m/z (FAB, 3-NOBA) 302 (20%, $[MH]^+$), 136 (70%, [MH-

$PhCH_2OC{=}O]^+$), 91 (100, $[PhCH_2]^+$), 77 (60%, $[Ph]^+$); Found 302.11872 $C_{17}H_{17}NO_3F$ requires 302.11925; $[\alpha]_D^{20}$ -29.7 (1.0, EtOAc), Lit[143] $[\alpha]_D^{20}$ –30 (1.3, EtOAc).

5.2.31 (S)-2-Benzotriazol-1-yl-1-benzyl-2-oxo-ethyl)-carbamic benzyl ester (114)

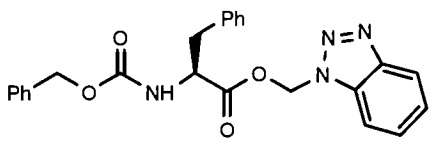


Benzotriazole (0.40g, 3.4 mmols) was dissolved in 'anhydrous' DCM (10ml) under an inert nitrogen atmosphere. This solution was cooled to 0°C in an ice bath and a solution of triethylamine (0.56ml, 4.1 mmols, and 1.2 equiv.) in 'anhydrous 'DCM (5ml) was added drop wise. After complete addition of the triethylamine, a solution of (*S*)-**113** (1.13g, 3.7mmols, 1.1 equiv.), in 'anhydrous' DCM (5ml), was added drop wise. The reaction was stirred for 30mins at 0°c then a further 1 hour at room temperature. After this time the reaction was quenched by the addition of HCl (1M, 5ml). The organic layer was separated and the aqueous layer re-extracted with further DCM (20ml). The combined organic layers were washed with H_2O (10ml), aqueous $NaHCO_3$ (5%, 10ml), H_2O (10ml), sat. NaCl (10ml), dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to provide the desired product as a white solid (0.92g, 69%).

R_f (DCM, 100%) 0.38; Mp 148-150°c; υ_{max}(nujol)/cm^{-1} 3311 (NH), 3029 (CH_{ar}), 1743 (C=O), 1688 (C=O, urethane), 1541 (C=O, urethane); δ_H ($CDCl_3$; 250MHz) 3.22 (1H, dd, J 14 and 7.7Hz, $CHC\underline{H}_aCH_b$), 3.48 (1H, dd, J 14 and 5.1 Hz, $CHCH_a\underline{H}_b$), 5.08 (2H, s, $C\underline{H}_2OCO$) 5.54 (1H, d, J 8Hz, N<u>H</u>), 6.04-6.13 (1H, m, $C\underline{H}CH_2$), 7.11–7.32 (10H, m, $C\underline{H}_{ar}$ x 10), 7.54 (1H, ddd, J 8.2, 7.1 and 1.1, $C\underline{H}_{bt}$), 7.68 (1H, ddd, J 8.2, 7.1 and 1.1, $C\underline{H}_{bt}$), 8.15 (1H, d, J 8.2Hz, $C\underline{H}_{bt}$) and 8.23 (1H, d, J 8.2Hz, $C\underline{H}_{bt}$); δ_C ($CDCl_3$; 63MHz) 38.7 ($\underline{C}H_2$), 55.5 ($\underline{C}H$), 67.1 ($\underline{C}H_2OCO$), 114.2 ($\underline{C}H_{bt}$), 120.3 ($\underline{C}H_{bt}$), 126.5 ($\underline{C}H_{bt}$), 127.3 ($\underline{C}H_{ar}$), 128.0 ($\underline{C}H_{ar}$), 128.2 ($\underline{C}H_{ar}$), 128.4 ($\underline{C}H_{ar}$), 128.6 ($\underline{C}H_{ar}$ x 2), 129.1 ($\underline{C}H_{ar}$ x 2), 130.7 ($\underline{C}H_{ar}$), 130.9 ($\underline{C}_{ar}$), 134.8 ($\underline{C}_{ar}$), 135.9 ($\underline{C}_{ar}$), 145.9 ($\underline{C}_{ar}$), 155.6 (OC=O) and 170.7 (COF); m/z (ESi +ve, 45kV) 423.2 (40%, $[MNa]^+$), m/z (FAB, 3-NOBA) 401 (20%,

$[MH]^+$), 136 (80%, $[MH- PhCH_2OC=O]^+$), 120 (30%, $[Bt]^+$), 91 (100, $[PhCH_2]^+$), 77 (60%, $[Ph]^+$); Found 401.16089 $C_{23}H_{21}N_4O_3$ requires 401.16137; $[\alpha]_D^{20}$ +20.2 (1.09, $CHCl_3$).

5.2.32 (*S*) -2-Benzyloxycarbonylamino-3-phenyl-propionic benzotriazol-1-ylmethyl ester (112)



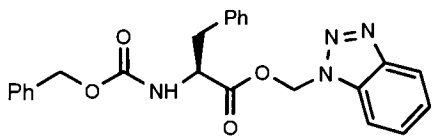
(*S*)-**114** (0.5g, 1.3mmols) and powdered potassium carbonate (15mol%, 25.9mg, and 0.2mmols) were suspended in acetonitrile (50ml- large volume required due to insolubility of **114)** and stirred vigorously. To this solution was added formaldehyde (1.05equivs, 0.106ml, and 1.4mmols) and the reaction was stirred at room temperature overnight. TLC indicated that the reaction had not gone to completion so a further volume of formaldehyde was added (1.05 equivs, 0.106ml, 1.4mmols) and the reaction mixture stirred for a further 16 hours at room temperature. The powdered potassium carbonate was removed by filtration and the solvent removed under reduced pressure to yield a yellow oil. Column chromatography on silica gel eluting with DCM:EtOAc (95:5) provided the desired product as a colourless glassy solid (288mg, 54%).

The chiral purity of **114** was determined to be 90% e.e. by normal phase HPLC eluting with Hexane:IPA (80:20) at 1ml/min^{-1} and 5°C.
Note: The 2' ester of **114** was not isolated but a very faint spot (Rf 0.57, DCM:EtOAc (95:5)) was detected.

R_f (DCM:EtOAc, 95:5) 0.43; R_t (*S*)-enantiomer 40.9mins, (*R*)-enantiomer 48.1mins; Mp 98-100°C; υ_{max}(thin film)/cm^{-1} 3323 (NH), 3052, 3032 (CH_{ar}), 2976, 2930 (CH), 1758 (C=O), 1716 (C=O, urethane), 1615 (C=C), 1541 (C=O, urethane), 1496, 1455; δ_H ($CDCl_3$; 250MHz) 3.01 (2H, d, J 5.9Hz, $CHC\underline{H}_2$), 4.64-4.72 (1H, m, $C\underline{H}CH_2$), 5.06 (2H, s, $C\underline{H}_2OCO$) 5.19 (1H, d, J 8.3Hz, N$\underline{H}$), 6.51 (1H, d, J 11.1Hz, $C\underline{H}_aH_bO$), 6.69-

6.74 (3H, m, CH_aH_bO + CH_{ar} overlapping), 6.90-7.08 (3H, m, $C\underline{H}_{ar}$ x 3) 7.31–7.39 (5H, m, $C\underline{H}_{ar}$ x 10), 7.43 (1H, ddd, J 8.1, 7.0 and 1.0Hz, $C\underline{H}_{bt}$), 7.52 (1H, ddd, J 8.2, 7.0 and 1.0 Hz, $C\underline{H}_{bt}$), 7.66 (1H, d, J 8.3Hz, $C\underline{H}_{bt}$) and 8.10 (1H, d, J 8.2Hz, $C\underline{H}_{bt}$); δ_C ($CDCl_3$; 63MHz) 37.5 ($\underline{C}H_2$), 54.5 ($\underline{C}H$), 67.0 ($\underline{C}H_2OCO$), 68.1 ($O\underline{C}H_2$), 109.9 ($\underline{C}H_{bt}$), 120.0 ($\underline{C}H_{bt}$), 124.5 ($\underline{C}H_{bt}$), 127.0 ($\underline{C}H_{ar}$), 128.0 ($\underline{C}H_{ar}$ x 2), 128.1 ($\underline{C}H_{ar}$ x 2), 128.3 ($\underline{C}H_{ar}$ x 2), 128.3 (CH_{ar}),128.7 ($\underline{C}H_{ar}$ x 2), 132.6 ($\underline{C}_{ar}$), 134.4 ($\underline{C}_{ar}$), 135.8 ($\underline{C}_{ar}$), 145.9 ($\underline{C}_{ar}$), 155.4 (OC=O) and 170.8 (C=O); m/z (ESi +ve, 45kV) 453.2 (100%, $[MNa]^+$), 431.2 (60%, $[MH]^+$; m/z (FAB, 3-NOBA) 453 (10%, $[MNa]^+$, 431 (100%, $[MH]^+$), 136 (30%, $[MH-PhCH_2OC{=}O]^+$), 91 (60, $[PhCH_2]^+$), 77 (35%, $[Ph]^+$); Found 431.17187 $C_{23}H_{21}N_4O_3$ requires 431.17193; $[\alpha]_D^{20}$ – 6.3 (1.27, $CHCl_3$).

5.2.33 (*S*)-2-Benzyloxycarbonylamino-3-phenyl-propionic benzotriazol-1-ylmethyl ester (112)

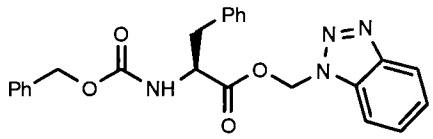


(*S*)-**113** (1g, 3.3mmols, 1 equiv), EDCI (0.63g, 3.3mmols, 1 equiv.), DIPEA (0.42g, 3.3mmols, 1 equiv) and 1-hydroxybenzotriazole (0.45g, 3.3mmols, 1 equiv.) were dissolved in 'anhydrous' DCM (50ml) and allowed to stir for 30 minutes at room temperature. 1-Hydroxymethylbenzotriazole **100** (0.492g, 3.3mols, 1 equiv.) was added and the solution stirred for a further 16 hours at room temperature. The reaction mixture was washed with H_2O (10ml), aqueous K_2CO_3 (10%, 10ml), H_2O (10ml) and brine. The organic layer was separated, dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to give a thick yellow oil. Column chromatography eluting with DCM:EtOAc (95:5) gave the desired product as a white solid (0.539g, 37%).

The chiral purity of (*S*)-**112** was determined to be 100% e.e. by normal phase HPLC eluting with Hexane: IPA (80:20) at 1ml/min^{-1} and 5°c.

Analysis comparable to **5.2.32** except R_t (*S*)- enantiomer 40.9mins, (*R*)-enantiomer not detected; m/z (FAB, 3-NOBA) Found 431.17295 $C_{23}H_{21}N_4O_3$ requires 431.17193; $[\alpha]_D^{20}$ – 8.9 (1.02, $CHCl_3$).

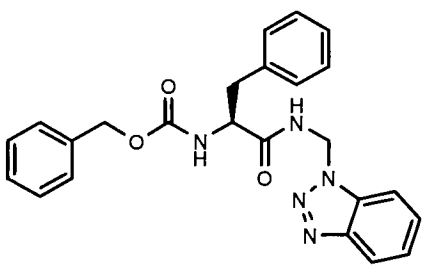
5.2.34 (*R/S*) -2-Benzyloxycarbonylamino-3-phenyl-propionic benzotriazol-1-yl methyl ester (112)



(*R/S*)-**103** (1g, 3.3mmols, 1 equiv), EDCI (0.634, 3.3mmols, 1 equiv.), DIPEA (0.42g, 3.3mmols, 1 equiv) and 1-hydroxybenzotriazole (0.446g, 3.3mmols, 1 equiv.) were dissolved in 'anhydrous' DCM (50ml) and allowed to stir for 30 minutes at room temperature. 1-Hydroxymethylbenzotriazole **100** (0.492g, 3.3mmols, 1 equiv.) was added and the solution stirred for a further 16 hours at room temperature. The reaction mixture was washed with H_2O (10ml), aqueous K_2CO_3 (10%, 10ml), H_2O (10ml) and brine. The organic layer was separated, dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to give a thick yellow oil. Column chromatography eluting with DCM: EtOAc (95:5) gave the desired product as a thick oil (0.350g, 25%).

The enantiomeric purity of (*R/S*)-**112** was determined to be 0% e.e. by normal phase HPLC eluting with hexane:IPA (80:20) at 1ml/min^{-1} and 5°C.
Analytical data comparable to **5.2.33** except m/z (FAB, 3-NOBA) Found 431.17185 $C_{23}H_{21}N_4O_3$ requires 431.17193.

5.2.35 (*S*)-{ 1-[(Benzotriazol-1-ylmethyl)-aminocarbonyl]-2-phenyl-ethyl}-carbamic acid benzyl ester.

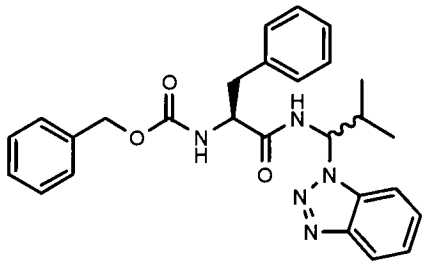


Commercially available Z-*L*-phenylalanine amide **116** (0.5g, 1.67mmols, 1equiv.) and 1-hydroxymethylbenzotriazole **100** (0.25g, 1.67mmols, 1 equiv.) were

dissolved in acetic acid (4ml) and refluxed for 6 hours. The acetic acid was removed under reduced pressure to give a yellow oil which was dissolved in DCM (10ml) and washed with Na_2CO_3 (10%, 6ml). The organic layer was separated, dried over $MgSO_4$ and the solvent removed to give a white solid. Purification by silica gel chromatography eluting with EtOAc:DCM (1:1) gave the desired compound as a white solid (0.237g, 33%).

M.p. 162°C R_f (EtOAc:DCM, 1:1) 0.53; υ_{max}(nujol)/cm^{-1} 3293 (NH), 1682 (C=O, urethane), 1628 (C=O, amide), 1605 (C=C) and 1537 (C=O, amide); δ_H ($(CD_3)_2SO$; 250MHz) 2.73 (1H, dd, J 13.6 and 10.0Hz, $CHC\underline{H}_aH_b$), 2.92 (1H, dd, J 13.6 and 4.7Hz, $CHC\underline{H}_aH_b$), 4.23-4.30 (1H, m, $C\underline{H}CH_2$), 4.95 (2H, s, $C\underline{H}_2O$), 6.03 (1H, dd, J 14.0 and 6.4Hz, $NHC\underline{H}_aH_b$), 6.07 (1H, dd, J 14.0 and 6.5Hz, $NHCH_a\underline{H}_b$), 7.00-7.37 (11H, m, Ph x10 + OCON$\underline{H}$), 7.44 (1H, ddd, J 8.2, 7.1 and 1.0Hz, $C\underline{H}_{bt}$), 7.57 (1H, ddd, J 8.2, 7.1 and 1.0Hz, $C\underline{H}_{bt}$), 7.96 (1H, d, J 8.4Hz, $C\underline{H}_{bt}$), 8.07 (1H, d, J 8.4Hz, $C\underline{H}_{bt}$) and 9.50 (1H, t, J 6.5Hz, $CH_2N\underline{H}$); δ_c ($(CD_3)_2SO$; 63MHz) 38.0 ($CH\underline{C}H_2$), 52.0 (NCH_2N), 57.1 ($\underline{C}HCH_2$), 66.2 ($\underline{C}H_2O$), 112.2 ($\underline{C}H_{bt}$), 119.9 ($\underline{C}H_{bt}$), 125.0 ($\underline{C}H_{bt}$), 127.1 ($\underline{C}H_{bt}$), 128.2 ($\underline{C}H_{ar}$), 128.3 ($C\underline{H}_{ar}$ x 2), 128.6 ($\underline{C}H_{ar}$), 128.8 ($\underline{C}H_{ar}$ x 2), 129.1 ($\underline{C}H_{ar}$ x 2), 129.9 ($C\underline{H}_{ar}$ x 2), 133.0 ($\underline{C}_{bt}$), 137.8 ($\underline{C}_{ar}$), 138.4 ($\underline{C}_{ar}$), 146.2 ($\underline{C}_{bt}$), 156.7 (C=O, urethane) and 173.4 (C=O, amide); m/z (ESi +ve, 45kV) 468.0 (10%, $[MK]^+$), 451.9 (100%, $[MNa]^+$), 429.9 (10%, $[MH]^+$); m/z (FAB, 3-NOBA) 430 (20%, $[MH]^+$), 120 (50%, $[BtH]^+$), 107 (60%, $[PhCH_2O]^+$), 91 (70%, $[PhCH_2]^+$)and 77 (60%, $[Ph]^+$); m/z (FAB, 3-NOBA) Found 430.18775 $C_{24}H_{24}N_5O_3$ requires 430.18789; $[\alpha]_D^{20}$ -12.3 (1.00, $CHCl_3$)

5.2.36 [1-(1-Benzotriazol-1-yl-2-methyl-propylcarbamoyl)-2-phenyl-ethyl]-carbamic acid benzyl ester.

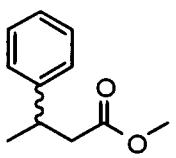


Commercially available Z-*L*-phenylalanine amide **116** (2.98g, 10mmol, 1 equiv.), freshly distilled *iso*-butyraldehyde (1.02g, 14.1mmols, 1.4 equivs.), benzotriazole (1.81g, 15.2mmols, 1.5equivs.) and a catalytic amount of *p*-toluenesulphonic acid (0.1g) were stirred in anhydrous toluene (30ml) for 30 minutes at room temperature. The reaction mixture was heated under reflux for 2 hours, using Dean Stark conditions to remove any water formed. After this time, TLC indicated that a substantial amount of amide remained and so further *iso*-butyraldehyde was added and refluxing continued for a further 2 hours. The solution was allowed to cool and the solvent removed under reduced pressure to yield a brown oil. The brown oil was dissolved in ethyl acetate (30ml), washed with K_2CO_3 (1M, 10ml x 2) and water (10ml x 2). The organic layer was separated, dried over $MgSO_4$ and the solvent removed under reduced pressure to yield brown oil. Further purification by column chromatography on silica gel eluting with Hexane:EtOAc (1:1) gave the desired product as a white foam (2.16g, 44%). The compound was obtained as a 50:50 mixture of diastereomers as confirmed by HPLC and integrals in ^{1}H NMR.

M.p. 183°C; R_f (Hexane:EtOAc, 1:1) 0.4; υ_{max}(IR card)/cm^{-1} 3293 (NH), 1681 (C=O, urethane), 1668 (C=O, amide) and 1537 (C=O, amide); δ_H ($(CD_3)_2SO$; 250MHz) 0.56 (1.5H, d, J 6Hz, $CH(CH_3)_3$), 0.58 (1.5H, d, J 6Hz, $CH(C\underline{H}_3)_3$), 1.00 (1.5H, d, J 6.5Hz, $CH(C\underline{H}_3)_3$), 1.16 (1.5H, d, J 6.5Hz, $CH(C\underline{H}_3)_3$), 2.52-2.79 (3H, m, $CHC\underline{H}_2$ + $C\underline{H}$(CH3)3 overlapping), 4.29-4.30 (1H, m, $C\underline{H}CH_2$), 4.96 (2H, s, $C\underline{H}_2O$), 6.24-6.37 (1H, m, $NHC\underline{H}(CH)N_{bt}$), 7.00-7.37 (11H, m, Ar$\underline{H}$ + OCON$\underline{H}$), 7.46 (1H, ddd, J 8.2, 7.1 and

1.0Hz, C<u>H</u>$_{bt}$), 7.55 (1H, ddd, J 8.2, 7.1 and 1.0Hz, C<u>H</u>$_{bt}$), 7.92 (1H, d, J 8.4Hz, C<u>H</u>$_{bt}$), 8.03 (1H, d, J 8.4Hz, C<u>H</u>$_{bt}$), 9.49 (0.5H, d, J 8.3Hz, CHN<u>H</u>) and 9.52 (0.5H, d, J 8.3Hz, CHN<u>H</u>); δ_c ($(CD_3)_2SO$; 63MHz) 18.4 (CH(<u>C</u>H$_3$)$_2$), 18.9, 19.1 (CH(<u>C</u>H$_3$)$_2$), 31.8, 31.9 (<u>C</u>H(CH$_3$)$_2$), 37.2, 37.8 (CH<u>C</u>H$_2$), 55.9, 56.2 (<u>C</u>HCH$_2$), 65.3, 65.4 (<u>C</u>H$_2$O), 68.7, 68.9 (NH<u>C</u>HN) 111.2, 111.3 (<u>C</u>H$_{bt}$), 119.2 (<u>C</u>H$_{bt}$), 124.2, 124.2 (<u>C</u>H$_{bt}$), 127.1 (<u>C</u>H$_{bt}$), 128.2 (<u>C</u>H$_{ar}$), 128.3 (C<u>H</u>$_{ar}$ x 2), 128.6 (<u>C</u>H$_{ar}$), 128.8 (<u>C</u>H$_{ar}$ x 2), 129.1 (<u>C</u>H$_{ar}$ x 2), 129.9 (C<u>H</u>$_{ar}$ x 2), 132.3, 132.6 (<u>C</u>$_{bt}$), 137.0, 137.1, 137.4, 137.7 (<u>C</u>$_{ar}$), 145.1, 145.2 (<u>C</u>$_{bt}$), 155.8, 155.9 (C=O, urethane) 172.2 and 172.3 (C=O, amide); m/z (ESi -ve, 45kV) 470.4 (100%, $[MH]^-$); m/z (FAB, 3-NOBA) 472 (50%, $[MH]^+$), 120 (50%, $[BtH]^+$), 107 (30%, $[PhCH_2O]^+$), 91 (70%, $[PhCH_2]^+$) and 77 (50%, $[Ph]^+$); m/z (FAB, 3-NOBA) Found 472.23442 $C_{27}H_{30}N_5O_3$ requires 472.23487; $[\alpha]_D^{20}$ -17.2 (1.00, $CHCl_3$).

5.2.37 (*R/S*) 3-Phenylbutyric acid methyl ester (104)



(*R/S*)-3-Phenylbutyric acid (4g, 24mmols) was dissolved in methanol (30ml) and a catalytic amount of conc. H_2SO_4 was added. The resulting mixture was refluxed for 8 hours and allowed to cool. The methanol was removed under reduced pressure and the crude oil dissolved in hexane (50ml). The solution was washed with $NaHCO_3$ (5%, 20ml x 3), H_2O (30ml) and the organic layer separated. The organic layer was dried over $MgSO_4$ and the solvent removed to give the desired product as a clear liquid (3.7g, 85%).

Chiral HPLC on a Chiracel OD-H column using an isocratic method eluting with 10% IPA and 90% Hexane at 20°c gave baseline separation of both enantiomers.

Rt (*R*)-enantiomer 4.3mins, (*S*)- enantiomer 6.7mins;υ_{max}(neat)/cm^{-1} 3084, 3062, 3029 (CH_{ar}), 2964, 2874 (CH), 1738 (C=O, ester) and 1603 (C=C); δ_H ($CDCl_3$; 200MHz) 1.39 (3H, d, J 7.0Hz, $CHC\underline{H}_3$), 2.62 (1H, dd, J 15.3 and 8.6Hz, $CHC\underline{H}_a H_b$), (1H, dd, J 15.3 and 6.6Hz, $CHCH_a\underline{H}_b$), 3.28-3.46 (1H, m, $C\underline{H}CH_2$), 3.70 (3H, s, $OC\underline{H}_3$) and 7.25-7.43 (5H, m, Ph); δ_c ($CDCl_3$; 63MHz) 20.8 ($CH\underline{C}H_3$), 35.5 ($\underline{C}H$), 41.8 ($\underline{C}H_2$), 50.4 ($O\underline{C}H_3$), 125.4 ($\underline{C}H_{ar}$), 125.7 ($\underline{C}H_{ar}$ x 2), 127.5 ($\underline{C}H_{ar}$ x 2), 144.7 ($\underline{C}_{ar}$) and 171.7 (C=O).

Note: A sample of (*S*)-3-Phenyl butyric acid methyl ester **104** was also prepared in a similar manner to use as a standard for Chiral HPLC.

5.3.0 Enzymatic studies on benzotriazole substrates

5.3.1 Screening of lipases for hydrolysis of 94a-e, 95d and 96.

Hydrolysis experiments were performed in glass tubes (2ml) with plastic stoppers. 0.9ml of a stock substrate solution (to give 0.003mmol per reaction *e.g.* for **94b** 0.8mg/ml) was added to 0.1ml of a stock enzyme solution (1mg/ml). The solutions were rotated for three hours at room temperature on a 360° blood rotator, after which time a 200µl sample was taken and quenched with 200µl of acetone. The resulting solution was centrifuged, to remove the precipitated enzyme, and 200µl of supernatant were diluted to 600 µl with CH_3CN:H_2O (50:50) and analysed by reverse phase HPLC. Control experiments contained all reagents except the enzyme.

HPLC conditions: Using equipment according to section **5.1.3** with a gradient method eluting with H_2O (0.1% TFA) and CH_3CN (0.1% TFA).

Time (mins)	%H_2O (0.1%TFA)	%CH_3CN (0.1%TFA)
0	70	30%
30	10	90
35	10	90
35.01	95	5

Specificity of HPLC method:

Analyte	R_t(mins)
Benzotriazole	4.8
94a	13.2
94b	16.1
94c	17.8
94d	19.1
94e	19.0
95d	20.8
96	17.5
p-NO_2-benzaldehyde	11.5

Stock substrate solutions: Prepared to give 0.003mmols of substrate per reaction (*i.e.* for **94b** concentration = 0.8mg/ml in 10% CH_3CN pH7 buffer)

Stock lipase/esterase solution: Prepared freshly prior to each hydrolysis experiment at a nominal concentration of 1mg/ml in pH7 buffer.

Sodium phosphate buffer solution (pH7)**:** Prepared in accordance with Methods in Enzymology[161]

5.3.2 Enzymatic hydrolysis of (+/-) 3-phenylbutyric acid benzotriazol-1-ylmethyl ester and (+/-) 3-Phenylbutyric acid methyl ester

Hydrolysis experiments were performed, in quadruplicate, in glass tubes (2ml) with plastic stoppers. 0.05ml of a stock substrate solution (50.1mg/ml for (*R/S*)-**105** and 30.0mg/ml for (*R/S*)-**104**) was added to 0.45ml of a stock enzyme solution. The solutions were rotated for a known time period at room temperature on a 360° blood rotator, after which time 1ml of acetonitrile was added. 2 samples were taken

immediately; the first a 0.5ml aliquot diluted to 1ml with further acetonitrile for analysis by reverse phase HPLC and the second a 0.25ml aliquot diluted to 1ml with IPA for analysis by normal phase chiral HPLC. The reverse phase HPLC method allowed determination of the extent of conversion (using the relative response factor (RRF) of the ester with respect to the 3-phenylbutyric acid) and the chiral method determined the e.e. of the remaining substrate. Control experiments contained all reagents except the enzyme

Reverse phase HPLC conditions: Using equipment according to section **5.1.3** with an isocratic method eluting with 55% H_2O (0.1% TFA) and 45% CH_3CN (0.1% TFA).

Specificity of HPLC method:

Analyte	**R_t (mins)**	**RRF**
Benzotriazole	3.2	-
3-Phenylbutyric acid	5.9	-
104	15.3	1.01
105	21.7	2.87

Normal phase HPLC conditions (*R/S*)**-105:** Using equipment according to section **5.1.3** on a Chiracel OD column using an isocratic method eluting with 55% IPA and 45% Hexane at 20°C.

Analyte	R_t (mins)
(*R*)-**105**	6.6
(*S*)-**105**	8.6

Normal phase HPLC conditions for (*R/S*)**-104:** Using equipment according to section **5.1.3** on a Chiracel OD-H column using an isocratic method eluting with 10% IPA and 90% Hexane at 20°C.

Analyte	R_t (mins)
(*R*)-**104**	4.3
(*S*)-**104**	6.7

5.3.3 Initial rate determination for hydrolysis of (*R/S*)-104 and (*R/S*)-105

Hydrolysis experiments were performed, in duplicate, using a Gilson SK233 system. 2ml of a stock substrate solution (1.0mg/ml for (*R/S*)-**105** and 0.6mg/ml for (*R/S*)-**104** in CH_3CN) was added, followed by 15ml of pH7 buffer. The hydrolysis was initiated by addition of 1ml of the stock enzyme solution (1mg/ml in pH 7 buffer).The solutions were magnetically stirred and sampled automatically after 2 minutes. Subsequent sampling occurred every five minutes for the first 30minutes and thereafter every 30minutes for 4 hours. At each sampling interval 250µl was removed, placed in a separate HPLC vial and quenched by the addition of 750µl of acetonitrile. HPLC analysis using the methods documented in section **5.3.2** determined the amount of conversion.

Note: For slower hydrolysis reactions sampling was performed at less frequent intervals over a longer analysis time.

5.3.4 α– Chymotrypsin hydrolysis of 117 and 118.

Hydrolysis experiments were performed in glass tubes (2ml) with plastic stoppers. 0.1ml of a stock substrate solution (1mg/ml in CH_3OH), 0.1ml of α–chymotrypsin solution (freshly prepared at 1mg/ml in distilled water), 0.1ml of 0.1M Tris HCl buffer at pH 7.8 and 0.7 ml of distilled H_2O were added. The solutions were rotated for 24 hours at room temperature on a 360° blood rotator, after which time the solution was analysed directly by reverse phase HPLC using a gradient method described in **Section 5.3.1.**

Specificity of HPLC method:

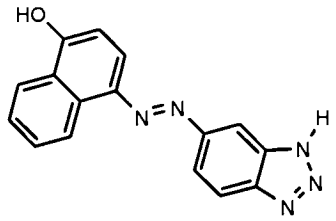
Analyte	R_t (mins)
Benzotriazole	4.8
Z-Phe-NH_2	13.4
103	14.3
118	15.7
112	17.1
117 – diastereomer 1	26.8
117 –diastereomer 2	27.0

5.4 Synthesis of SERRS active benzotriazole dyes

5.4.1 General procedure for phenolic monoazo benzotriazole dyes 119 and 120

5-aminobenzotriazole (1.1equiv.) was dissolved in a solution of 2ml of 36% (v/v) hydrochloric acid solution and 3ml of iced water. The resulting solution was cooled to 0°C and a cooled aqueous solution of sodium nitrate (1.2 equiv.) was added dropwise until an immediate blue/black colour was observed on starch/iodine paper (A small volume of the reaction solution was removed and tested). The diazotised aminobenzotriazole solution was added dropwise over 30mins to a stirred cooled solution of the appropriate phenol component (1.0 equiv) dissolved in sodium hydroxide solution (1.0M, 15ml) with sodium acetate added (10g) and acetone (15ml). The pH was monitored and maintained >7.0 by addition of further sodium hydroxide solution (1M) if required. The deeply coloured solution was maintained at 0°C for 30mins and allowed to stir at room temperature for a further 1hour. The pH of the solution was adjusted to 7.0 using 1M HCl and the precipitate formed collected by filtration. The precipitate was washed with saturated KCl (10ml x 2), 10% aq. acetic acid (10ml x 2) and H_2O. The crude precipitate was dissolved in DMF and precipitated by addition of water. The coloured product was filtered off and dried under reduced pressure over P_2O_5. The crude product was then purified by trituration in dichloromethane and ether (if required) to give the desired product as a highly coloured powder.

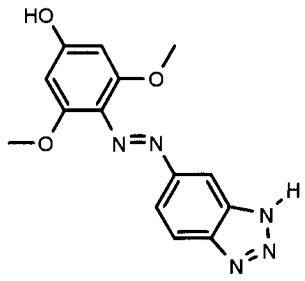
5.4.2 4-(1*H*-benzotriazol-6-ylazo)-napthalen-1-ol (120)



The general procedure outlined in section **5.4.1** was followed with 5-aminobenzotriazole (1g, 7.63mmols), sodium nitrate (0.58g, 9.16mmols) and α–Naphthol (1g, 6.94 mmols) to give the title compound as deep red powder (1.3g, 65%). The purity of the azo dye obtained was determined by HPLC as >99% eluting on a gradient of 95%H_2O to 95% CH_3CN over 30mins.

R_f (EtOAc:MeOH:NH_3 85:15:1%) 0.22; R_t 18.2 mins; Mp 224-226°C (dec.); υ_{max}(nujol)/cm^{-1} 1612 (C=C), 1559 (C=C), 1519; δ_H ($(CD_3)_2SO$; 250MHz) 7.02 (1H, d, J 8.7Hz, C<u>H</u>$_{ar}$), 7.59 (1H, ddd, J 8.2, 6.9 and 1.5Hz, C<u>H</u>$_{ar}$), 7.73 (1H, ddd, J 8.2, 6.9 and 1.5 Hz, C<u>H</u>$_{ar}$), 8.00 (1H, d, J 8.7Hz, C<u>H</u>), 8.04 (1H, dd, J 8.9 and 0.8Hz, C<u>H</u>$_{bt}$), 8.11 (1H, dd, J 8.9 and 1.3Hz, C<u>H</u>$_{bt}$), 8.23 (1H, d 8.4Hz, C<u>H</u>$_{ar}$), 8.38 (1H, br s, C<u>H</u>$_{bt}$) and 8.89 (1H, br d, 8.4Hz, C<u>H</u>$_{ar}$); δ_C ($(CD_3)_2SO$; 63MHz) 110.2 (CH$_{ar}$), 112.4, 115.9 and 118.4 (3 x <u>C</u>H$_{bt}$), 122.8, 122.8, 122.9 (3x <u>C</u>H$_{ar}$), 125.2 (<u>C</u>$_{ar}$), 125.9 (<u>C</u>H$_{ar}$), 128.5 (<u>C</u>H$_{ar}$), 129.5, 133.2, 143.0, 143.4, 149.7, 154.2 (6 x C$_{ar}$); m/z (ESi -ve, 45Kv) 288 (100%, $[MH]^-$); m/z (FAB, 3-THIO) 290 (25%, $[MH]^+$), Found 290.10408 $C_{16}H_{12}N_5O$ requires 290.10419

5.4.3 4-(1*H*-benzotriazol-6-ylazo)-3-5-dimethoxyphenol.



The general procedure outlined in section **5.4.1** was followed with 5-aminobenzotriazole (1g, 7.63mmols), sodium nitrate (0.58g, 9.16mmols) and 3,5-dimethoxy phenol (1.07g, 6.94 mmols) to give the title compound as deep red powder (1.2g, 54%). The purity of the azo dye obtained was determined by HPLC as >99% eluting on a gradient of 95% H_2O to 95% CH_3CN over 30mins.

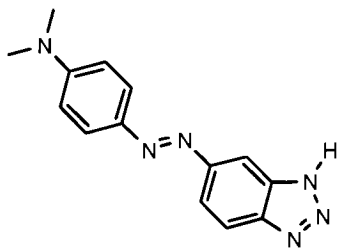
R_f (EtOAc:MeOH:NH_3, 85:15:1%) 0.32; R_t 18.5mins; Mp 216°-219°C (dec.); υ_{max}(nujol)/cm^{-1} 1612 (C=C), 1559 (C=C), 1519; δ_H ($(CD_3)_2SO$; 250MHz) 3.79 (6H, s, $(OC\underline{H}_3)_2$), 6.08 (2H, broad s, $C\underline{H}_{ar}$), 7.77 (1H, dd, J 8.9 and 1.7Hz, $C\underline{H}_{bt}$), 7.98 (1H, dd, J 8.9 and 0.7Hz, $C\underline{H}_{bt}$), 8.05 (1H, dd, J 1.7 and 0.7Hz, $C\underline{H}_{bt}$); δ_C ($(CD_3)_2SO$; 63MHz) 93.9 (2 x CH_{ar}), 111.4, 116.1, 117.0 (3x $\underline{C}H_{bt}$), 122.0 (C_{ar}), 133.2, 143.0 (2 x C_{bt}), 153.1 (C_{ar}), 157.4 (2 x C_{ar}), 159.2 (C_{ar}); m/z (ESi -ve, 45Kv) 298 (100%, $[MH]^-$); m/z (FAB, 3-THIO) 300 (25%, $[MH]^+$), Found 300.10958 $C_{14}H_{14}N_5O_3$ requires 300.10967.

5.4.4 General procedure for preparation of *N'N'*-Dimethyl monoazo benzotriazole dyes

5-aminobenzotriazole (1.0equiv.) was dissolved in a solution of 1ml of 36% (v/v) hydrochloric acid solution and 1.5ml of iced water. The resulting solution was cooled to 0°C and a cooled aqueous solution of sodium nitrate (1.2 equiv.) was added drop wise until an immediate blue/black colour was observed on starch/iodine paper (A small

volume of the reaction solution was removed for testing). The diazotised aminobenzotriazole solution was added drop wise over 30mins to a stirred cooled solution of the appropriate *N'N'*-Dimethyl component (1.0-1.1 equiv) dissolved in sodium acetate buffer (1.0M, 20ml, pH=6.0) and acetone (25ml). The deeply coloured solution was maintained at 0°C for 30mins after which time 1M NaOH was added until the solution changed colour and stirring was continued for a further 30mins. The pH of the solution was adjusted to pH7.0 using 1M HCl and the precipitate formed collected by filtration. The precipitate was washed with saturated KCl (25ml x 2), 10% aq. acetic acid (25ml x 2) and H_2O. The wet cake was dried under vacuum over P_2O_5 to constant weight, and then purified by trituration in dichloromethane, methanol and ether to give the desired product as a highly coloured powder.

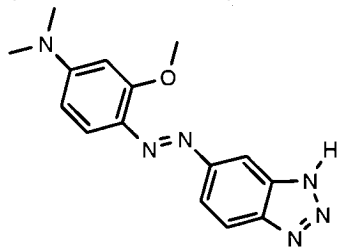
5.4.5 [4-(1*H*-Benzotriazol-6-yl-azo)-phenyl]-dimethylamine (121)



The general procedure outlined in section **5.4.4** was followed with 5-aminobenzotriazole (0.75g, 5.6mmol), sodium nitrate (0.51g, 6.7mmol) and *N'N'*-Dimethyl aniline (0.67g, 5.6 mmol) was followed and gave the title compound as an orange powder (1.29g, 86%).

R_f (EtOAc: MeOH, 99:1) 0.53; Mp 212-214°C (dec.); υ_{max}(nujol)/cm^{-1}, 1626(C=C), 1599 (C=C); δ_H ($(CD_3)_2SO$; 360MHz), 3.19 (6H, s, N($C\underline{H}_3)_2$), 6.86 (2H, d, J 9.2Hz, 2x$C\underline{H}_{ar}$), 7.86 (2H, d, J 9.2Hz, 2x$C\underline{H}_{ar}$), 7.96 (1H, dd, J 8.9 and 1.6Hz, $C\underline{H}_{bt}$), 8.01 (1H, d, J 8.9Hz, $C\underline{H}_{bt}$), 8.23 (1H, s, $C\underline{H}_{bt}$); δ_C ($(CD_3)_2SO$; 63MHz) 40.0 (N($\underline{C}H_3)_2$), 110.7 ($\underline{C}H_{bt}$), 111.7 (2x$\underline{C}H_{ar}$), 115.7 ($\underline{C}H_{bt}$), 118.1 ($\underline{C}H_{bt}$), 124.9 (2x $\underline{C}H_{ar}$), 140.1, 140.2, 142.6, 150.1 and 152.6 (5 x $\underline{C}_{ar}$); m/z (ESi- 45Kv) 265.1 (100%, $[MH]^+$); m/z (FAB, THIO) Found 267.13583, $C_{14}H_{15}N_6$ requires 267.13582.

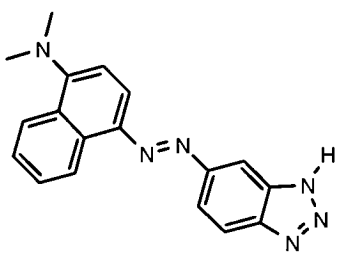
5.4.6 [4-(1*H*-Benzotriazol-6-ylazo)-3-methoxy-phenyl]-dimethyl-amine (122)



The general procedure outlined in Section **5.4.4** was followed with 5-aminobenzotriazole (1.5g, 11.2mmols), sodium nitrate (0.87g, 12.6mmols) and 3-dimethylaminoanisole (1.539g, 11.2 mmols) was followed to give the title compound as deep red powder (2.4g, 78%).

R_f (EtOAc, 100%) 0.18; Mp 116-118°C (dec.); υ_{max}(nujol)/cm^{-1} 1601 (C=C), 1556 (C=C); δ_H ($(CD_3)_2SO$; 250MHz), 3.07 (6H, s, $N(C\underline{H}_3)_2$), 3.97 (3H, s, $OC\underline{H}_3$), 6.33 (1H, d, J 2.4Hz, $C\underline{H}_{ar}$), 6.40 (1H, dd, J 9.2 and 2.5Hz, $C\underline{H}_{ar}$), 7.69 (1H, d, J 9.2Hz, $C\underline{H}_{ar}$), 7.86 (1H, dd, J 8.9 and 1.6Hz, $C\underline{H}_{bt}$), 7.91 (1H, dd, J 8.9 and 0.8Hz, $C\underline{H}_{bt}$), 8.14 (1H, dd, J 1.6 and 0.8Hz, $C\underline{H}_{bt}$); δ_C ($(CD_3)_2SO$; 63MHz) 40.2 ($N(\underline{C}H_3)_2$), 56.7 ($O\underline{C}H_3$), 95.8 ($\underline{C}H_{ar}$), 105.5 ($\underline{C}H_{ar}$), 110.4 ($\underline{C}H_{bt}$), 116.5 ($\underline{C}H_{bt}$), 118.1 ($\underline{C}H_{bt}$), 119.2 ($\underline{C}H_{ar}$), 133.2, 140.4, 140.9, 151.6, 155.2, 160.1 (6 x $\underline{C}_{ar}$); m/z (ESi+, 45Kv) 296.9 (100%, $[MH]^+$); m/z (FAB, NOBA) 297 (50%, $[MH]^+$); Found 297.14648, $C_{15}H_{17}N_6O$ requires 297.14639.

5.4.7 [4-(1*H*-Benzotriazol-6-ylazo)-naphthalen-1-yl]-dimethyl-amine (123)

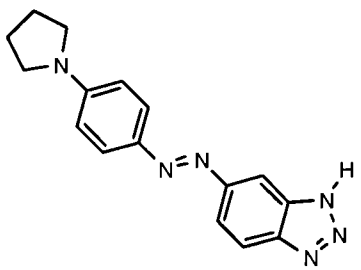


The general procedure outlined in section **5.4.4** was followed with 5-aminobenzotriazole (0.53g, 3.9mmols), sodium nitrate (0.32g, 4.7mmols) and *N'N'*-

Dimethyl-napthylamine (0.7mls, 4.3mmols) to give the title compound as a bright orange powder (0.91g, 74%).

R_f (EtOAc: MeOH 99:1) 0.67; Mp 210-212°C (dec.); υ_{max}(nujol)/cm^{-1} 1571 (C=C), 1507 (C=C), 1323; δ_H ($(CD_3)_2SO$; 250MHz), 2.98 (6H, s, N($C\underline{H}_3$)$_2$), 7.20 (1H, d, J 8.4Hz, $C\underline{H}_{ar}$), 7.64 (1H, ddd, J 8.1, 6.8 and 1.5Hz, $C\underline{H}_{ar}$), 7.72 (1H, ddd, J 8.1, 6.8 and 1.5Hz, $C\underline{H}_{ar}$), 7.91 (1H, d, J 8.4Hz, $C\underline{H}_{ar}$), 8.03 (1H, d, J 8.9Hz, $C\underline{H}_{bt}$), 8.12 (1H, dd, J 8.9 and 1.5Hz, $C\underline{H}_{bt}$), 8.21 (1H, d, J 8Hz, $C\underline{H}_{ar}$), 8.48 (1H, distorted s, $C\underline{H}_{bt}$), 9.00 (1H, d, J 8Hz, $C\underline{H}_{ar}$); δ_C ($(CD_3)_2SO$; 63MHz) 44.5 (N($\underline{C}H_3$)$_2$), 112.6 ($\underline{C}H_{bt}$), 113.3 ($\underline{C}H_{ar}$), 115.7 ($\underline{C}H_{bt}$), 117.8 ($\underline{C}H_{bt}$), 123.4, 124.7, 125.7, 125.9 and 127.2 (5 x $\underline{C}H_{ar}$), 127.5, 132.6, 140.6, 140.7, 141.5, 150.1 and 154.3 (7 x $\underline{C}_{ar}$); m/z (ESi +ve) 317.2 (100%, $[MH]^+$); m/z (FAB, NOBA) Found 317.15110 $C_{18}H_{17}N_6$ requires 317.15147

5.4.8 (1*H*-Benzotriazol-6-yl)-(4-pyrrolidin-1-yl-napthalen-1-yl)-diazene (124)



The general procedure outlined in section **5.2.1** was followed with 5-aminobenzotriazole (0.99g, 7.4mmols), sodium nitrate (0.61g, 8.9mmols) and 1-phenyl pyrollidine (1.2g, 8.1mmols) to give the title compound as a dark orange/brown powder (1.32g, 61%).

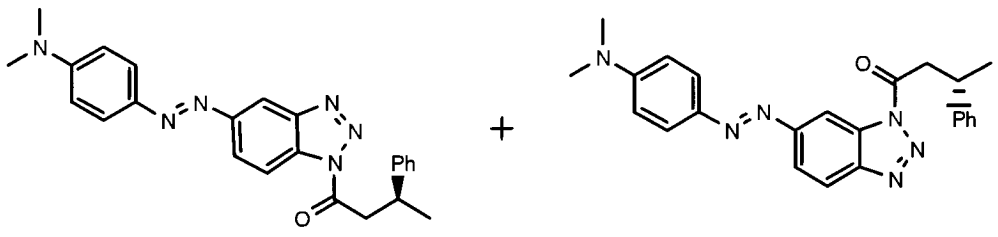
R_f (EtOAc: MeOH 99:1); Mp 224-226°C(dec.); υ_{max}(nujol)/cm^{-1} 1612 (C=C), 1559 (C=C), 1519; δ_H ($(CD_3)_2SO$; 250MHz) 1.98 (4H, m, 2 x $C\underline{H}_2CH_2N$), 3.42 (4H, m, 2 x $CH_2C\underline{H}_2N$), 6.67 (2H, d, J 9.1Hz, 2 x $C\underline{H}_{ar}$), 7.75 (1H, dd, J 8.9 and 1.7Hz, $C\underline{H}_{bt}$), 7.80 (2H, d, J 9.1Hz, 2 x $C\underline{H}_{ar}$), 7.84 (1H, dd, J 8.9 and 0.7Hz, $C\underline{H}_{bt}$), 8.17 (1H, dd, J 1.6 and 0.6Hz $C\underline{H}_{bt}$); δ_C ($(CD_3)_2SO$; 63MHz) 25.9 (2 x $\underline{C}H_2CH_2N$), 48.3 (2 x $CH_2\underline{C}H_2N$), 112.4

(2 x $\underline{C}H_{ar}$), 112.7, 116.5 and 116.7 (3 x $\underline{C}H_{bt}$), 125.6 (2 x $\underline{C}H_{ar}$), 142.9, 143.2, 143.4, 149.7, 150.5 (5 x C_{ar}); m/z (ESi +ve, 45Kv) 293.1 (100%, $[MH]^+$); m/z (FAB, 3-NOBA) 293 (62%, $[MH]^+$), Found 293.15147 $C_{16}H_{17}N_6$ requires 293.15142.

5.4.9 General procedure for acylation of *N'N* Dimethyl and pyrollidine monoazo benzotriazole dyes

The azo dye (1.0equiv.) **121-124** was suspended in 'anhydrous' DCM (see individual experiments for volume used) under an inert atmosphere of nitrogen and cooled to 0°C in an ice bath. To this stirred suspension was added a solution of triethylamine (1.2equiv.) in 1-2ml of 'anhydrous' DCM followed by the drop wise addition of a solution of the 'appropriate' acid chloride (1.1 equiv.) in 1-2ml of 'anhydrous ' DCM. Upon addition of the acid chloride the monoazo benzotriazole dye dissolved in the DCM and the reaction was stirred at 0°C for an hour. After this time the reaction was allowed to stir for a further 30 minutes while warming to room temperature. The reaction was quenched by addition of 5ml of 2M HCl, and an additional volume of DCM was added. The organic layer was separated, washed with H_2O, re-separated, dried over $MgSO_4$ and filtered. The solvent was removed under reduced pressure and the crude mixture of regioisomers purified by column chromatography, HPLC or trituration as appropriate.

5.4.10 1-[5-(4-Dimethylamino-phenylazo)-benzotriazol-1-yl]-3-(*S*)-phenyl- butan-1-one (137A) and 1-[6-(4-Dimethyl amino-phenylazo)-benzotriazol-1-yl]-3-(*S*)-phenyl-butan-1- one (137B)



A **B**

The general procedure (**5.4.9**) was followed with **121** (118.4mg, 0.44mmols) in 10ml of 'anhydrous' DCM. Triethylamine (53.8mg, 0.53mmols, 1.2 equiv.) and (*S*)-3-Phenylbutyryl chloride **106** (89mg, 0.49mmols, 1.1 equiv.) were added. The title compound was furnished as a thick dark red oil after column chromatography on silica eluting with DCM as a mixture of the two regioisomers (104mg, 57%) in the ratio 48:52 (**A**:**B**, respectively by NMR). The regioisomers were separated (baseline resolution not achieved) by prep-HPLC using a Biotage Flex system eluting with 87% CH_3CN / 13% H_2O. Crystals suitable for x-ray crystallography of **137B** were grown from acetonitrile; the crystals grown of **137A** did not diffract sufficiently to allow a crystal structure to be determined

137A

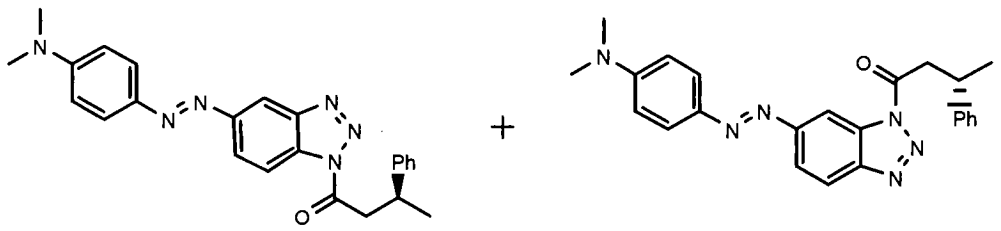
R_f (DCM) 0.58; R_t 7.3mins; Mp 153-155°C; υ_{max}(thin film)/cm^{-1} 3028 (CH_{ar}), 2963, 2928, 2873 (CH), 1739 (C=O), 1606 (C=C); δ_H ($CDCl_3$; 250MHz) 1.50 (3H, d, J 6.5Hz, $C\underline{H}_3$), 3.15 (6H, s, $N(C\underline{H}_3)_2$), 3.66-3.75 (2H, m, $C\underline{H}_2CH$), 3.80-3.87 (1H, m, $C\underline{H}CH_2$), 6.80 (2H, d , J 9.3Hz, 2 x $C\underline{H}_{ar}$), 7.24-7.39 (5H, m, Ar$\underline{H}$), 7.95 (2H, d, J 9.2Hz, 2 x $C\underline{H}_{ar}$), 8.24 (1H, dd, J 8.8 and 1.7Hz, $C\underline{H}_{bt}$), 8.32 (1H, dd, J 8.8 and 0.7Hz, $C\underline{H}_{bt}$), and 8.54 (1H, dd, 1.7 and 0.6Hz, $C\underline{H}_{bt}$); δ_c ($CDCl_3$; 63MHz) 22.0 ($\underline{C}H_3$), 36.0 ($\underline{C}H$), 40.2 ($N(\underline{C}H_3)_2$), 43.5 ($\underline{C}H_2$), 111.4 (2 x $\underline{C}H_{ar}$), 114.3, 114.3 (2 x $\underline{C}H_{bt}$), 124.3 ($\underline{C}H_{bt}$), 125.3 (2 x $\underline{C}H_{ar}$), 126.6 ($\underline{C}H_{ar}$), 126.8 (2 x $\underline{C}H_{ar}$), 128.6 (2 x $\underline{C}H_{ar}$), 131.2, 143.2, 144.9, 146.9, 151.6 and 152.7 (6 x $\underline{C}$ar) 170.9 (C=O); m/z (ESi +ve, 45Kv) 413 (100%, $[MH]^+$); m/z (FAB, NOBA) Found 413.20907 $C_{24}H_{25}N_6O$ requires 413.20898.

137B

R_f (DCM) 0.58; R_t 6.5mins; Mp 161-162°C; υ_{max}(thin film)/cm^{-1} 3028 (CH_{ar}), 1740 (C=O), 1606 (C=C); δ_H ($CDCl_3$; 250MHz) 1.50 (3H, d, J 6.5Hz, $C\underline{H}_3$), 3.15 (6H, s, $N(C\underline{H}_3)_2$), 3.67-3.75 (2H, m, $C\underline{H}_2CH$), 3.81-3.88 (1H, m, $C\underline{H}CH_2$), 6.80 (2H, d, J 9.2Hz, 2 x $C\underline{H}_{ar}$), 7.21-7.40 (5H, m, Ar$\underline{H}$), 7.96 (2H, d, J 9.2Hz, 2 x $C\underline{H}_{ar}$), 8.07 (1H, dd, J 8.8 and 1.7Hz, $C\underline{H}_{bt}$), 8.16 (1H, dd, J 8.8 and 0.7Hz, $C\underline{H}_{bt}$), 8.66 (1H, dd, 1.7 and 0.6Hz,

$C\underline{H}_{bt}$); δ_c ($CDCl_3$; 63MHz) 22.5 ($\underline{C}H_3$), 36.5 ($\underline{C}H$), 40.7 ($N(\underline{C}H_3)_2$), 44.0 ($\underline{C}H_2$), 108.7 ($\underline{C}H_{bt}$), 111.9 (2 x $\underline{C}H_{ar}$), 120.6 and 121.3 (2 x $\underline{C}H_{bt}$), 126.1 (2 x $\underline{C}H_{ar}$), 127.1 ($\underline{C}H_{ar}$), 127.3 (2 x $\underline{C}H_{ar}$), 129.1 (2 x $\underline{C}H_{ar}$), 132.3, 144.0, 145.6, 146.6, 153.4 and 154.9 (6 x $\underline{C}_{ar}$), 171.4 (C=O); m/z (ESi +ve, 45Kv) 413 (100%, $[MH]^+$); m/z (FAB, NOBA) Found 413.20903 $C_{24}H_{25}N_6O$ requires 413.20898.

5.4.11 1-[5-(4-Dimethyl amino-phenylazo)-benzotriazol-1-yl]-3-(*R*)-phenyl-butan-1-one (137A) and 1-[6-(4-Dimethylamino-phenylazo)-benzotriazol-1-yl]-3 (*R*)-phenyl-butan-1-one (137B)



A **B**

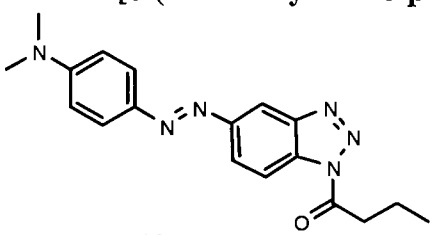
The general procedure outlined in section **5.4.9** was followed with **121** (99.3mg, 0.37mmols) in 10ml of 'anhydrous' DCM. Triethylamine (42mg, 0.41mmols) and (*R*)-3-Phenylbutyryl chloride **106** (72mg, 0.39mmols) were added. The title compound was furnished as a thick dark red oil after column chromatography on silica eluting with DCM as a mixture of the two regioisomers (104mg, 68%) in the ratio 48:52 by NMR of (A:B). The two regioisomers were not separated by HPLC.

Analysis identical to (**5.4.10**) except;

δ_H ($CDCl_3$; 250MHz) 1.54 (1.5H, d, J 6.3Hz, $C\underline{H}_3$, one regioisomer), 1.57 (1.5H, d, J 6.3Hz, $C\underline{H}_3$, one regioisomer), 3.19 (3H, s, $N(C\underline{H}_3)_2$, one regioisomer), 3.20 (3H, s, $N(C\underline{H}_3)_2$, one regioisomer), 3.66-3.75 (2H, m, $C\underline{H}_2CH$, both regioisomers), 3.80-3.87 (1H, m, $C\underline{H}CH_2$, both regioisomers), 6.84 (2H, d, J 8.6Hz, 2 x $C\underline{H}_{ar}$, both regioisomers), 7.33-7.42 (5H, m, Ar$\underline{H}$), 7.99 (2H, d, J 8.6Hz, 2 x $C\underline{H}_{ar}$, both regioisomers), 8.09 (0.5H, dd, J 8.8 and 1.7Hz, $C\underline{H}_{bt}$, **B**), 8.19 (0.5H, dd, J 8.8 and 0.7Hz, $C\underline{H}_{bt}$, **B**), 8.27 (0.5H, dd,

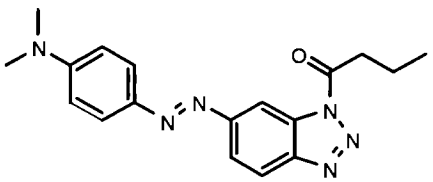
J 8.8 and 1.7Hz, C$\underline{H}_{bt}$, **A**), 8.35 (0.5H, dd, J 8.8 and 0.7Hz, C$\underline{H}_{bt}$, **A**), 8.58 (0.5H, dd, J 1.7 and 0.6Hz, C$\underline{H}_{bt}$, **A**) 8.69 (0.5H, dd, J 1.7 and 0.6Hz, C$\underline{H}_{bt}$, **B**); δ_c ($CDCl_3$; 63MHz) 21.9, 22.0 (C$\underline{H}_3$, both regioisomers), 35.8, 36.0 (C$\underline{H}$, both regioisomers), 40.2 (N(C$\underline{H}_3$)$_2$, both regioisomers), 43.4, 43.5 (C$\underline{H}_2$, both regioisomers), 108.7 (C$\underline{H}_{bt}$, **A**), 111.4 (2 x C$\underline{H}_{ar}$, one regioisomer), 111.5 (2 x C$\underline{H}_{ar}$, one regioisomer), 114.3, 114.4 (2 x C$\underline{H}_{bt}$, **A**), 120.7 and 121.4 (2 x C$\underline{H}_{bt}$, **B**), 124.5 (C$\underline{H}_{bt}$, **A**), 125.3 (2 x C$\underline{H}_{ar}$), 126.2 (2 x C$\underline{H}_{ar}$), 126.8 (C$\underline{H}_{ar}$, both regioisomers), 127.1 (2 x C$\underline{H}_{ar}$, both regioisomers), 128.9 (2 x C$\underline{H}_{ar}$, both regioisomers), 131.1, 132.4, 143.2, 144.1, 145.0, 145.5, 146.6, 147.0, 151.6, 152.7, 153.5 and 155.1 (12 x C$\underline{}_{ar}$, both regioisomers), 170.9 and 171.0 (2 x C=O, both regioisomers); m/z (ESi+, 45kV) 413.1 (100%, $[MH]^+$); m/z (FAB, NOBA) Found 413.20894 $C_{24}H_{25}N_6O$ requires 413.20898.

5.4.12 1-[5-(4-Dimethylamino-phenylazo)-benzotriazol-1-yl]-butan-1-one (130) and 1-[6-(4-Dimethylamino-phenylazo)-benzotriazol-1-yl]-butan-1-one (131)



130

+



131

The general procedure outlined in Section **5.4.9** was followed with **121** (136.4mg, 0.51mmols) in 10ml of 'anhydrous' DCM. Triethylamine (80µl, 0.57mmols) and butyryl chloride (59µl, 0.56mmols) were added. The title compounds were furnished as a red oil after column chromatography on silica eluting with DCM (121mg, 75%). The ratio of the regioisomers was calculated by comparison of the integrals at 8.56 and 8.72 p.p.m (in the crude mixture) and determined to be 52:48 (**131**:**130**). The two regioisomers were separated by HPLC on Biotage Flex system eluting with 75% CH_3CN: 25% H_2O.

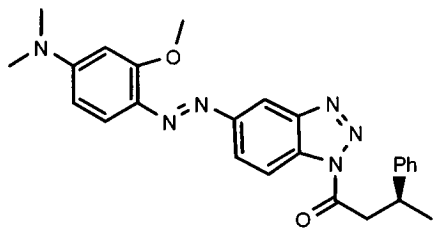
130

R_f (DCM, 100%) 0.42; R_t 4.3mins; Mp 184-185°C; υ_{max}(thin film)/cm^{-1} 2963, 2927(CH), 1740 (C=O), 1601 (C=C) and 1517 (C=C); δ_H ($CDCl_3$; 360MHz) 1.16 (3H, t, J 7.4Hz, $C\underline{H}_3$), 2.00 (2H, qt overlapping, J 7.5Hz and 7.5Hz, $CH_3C\underline{H}_2CH_2$), 3.15 (6H, s, $N(C\underline{H}_3)_2$), 3.46 (2H, t, J 7.5Hz, $CH_3CH_2C\underline{H}_2$), 6.81 (2H, d, J 9.2Hz, 2 x $C\underline{H}_{ar}$), 7.97 (2H , d, J 9.2Hz, 2 x $C\underline{H}_{ar}$), 8.28 (1H, dd, J 8.8 and 1.7Hz, $C\underline{H}_{bt}$), 8.37 (1H, dd, J 8.8 and 0.6Hz, $C\underline{H}_{bt}$), 8.56 (1H, dd, J 1.7 and 0.6Hz, $C\underline{H}_{bt}$); δ_c ($CDCl_3$; 63MHz) 14.1 ($\underline{C}H_3$), 18.4 ($CH_3\underline{C}H_2CH_2$), 37.7 ($CH_3CH_2\underline{C}H_2$), 40.7 ($N(\underline{C}H_3)_2$), 111.9 (2 x $\underline{C}H_{ar}$), 114.9 (CH_{bt}), 114.9 ($\underline{C}H_{bt}$), 124.8 ($\underline{C}H_{bt}$), 125.8 (2 x $\underline{C}H_{ar}$), 131.8, 143.8, 147.4, 152.1, 153.2 (5 x $\underline{C}_{ar}$), 172.9 (C=O); m/z (ESi +ve, 45Kv) 337 (100%, $[MH]^+$); m/z (FAB, 3-NOBA) 337 (65%, $[MH]^+$), 307 (70%, $[MH\text{-}CH_2CH_3]^+$), 267 (10%, $[MH\text{-}COCH_2CH_2CH_3]^+$); Found (FAB) 337.17807 $C_{18}H_{21}N_6O_1$ requires 337.17768.

131

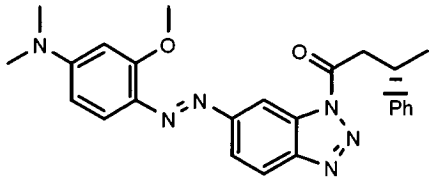
R_f (DCM, 100%) 0.44; R_t 4.1mins; Mp 178-179°C; υ_{max}(thin film)/cm^{-1} 2964, 2927 (CH), 1740. (C=O), 1601 (C=C) and 1517(C=C); δ_H ($CDCl_3$; 360MHz) 1.16 (3H, t, J 7.5Hz, $C\underline{H}_3$), 2.01 (2H, tq overlapping, J 7.5Hz and 7.5Hz, $CH_3C\underline{H}_2CH_2$), 3.14 (6H, s, $N(C\underline{H}_3)_2$), 3.42 (2H, t, J 7.5Hz, $CH_3CH_2C\underline{H}_2$), 6.82 (2H, d, J 9.3Hz, 2 x $C\underline{H}_{ar}$), 7.99 (2H, d, J 9.3Hz, 2 x $C\underline{H}_{ar}$), 8.10 (1H, dd, J 8.8 and 1.7Hz, $C\underline{H}_{bt}$), 8.18 (1H, dd, J 8.8 and 0.7Hz, $C\underline{H}_{bt}$), 8.72 (1H, dd, J 1.7 and 0.7Hz, $C\underline{H}_{bt}$); δ_c ($CDCl_3$; 63MHz) 14.1 ($\underline{C}H_3$), 18.3 ($CH_3\underline{C}H_2CH_2$), 37.8 ($CH_3CH_2\underline{C}H_2$), 40.8 ($N(\underline{C}H_3)_2$), 108.8 ($\underline{C}H_{bt}$), 112.1 (2 x $\underline{C}H_{ar}$), 120.6 ($\underline{C}H_{bt}$), 121.1 ($\underline{C}H_{bt}$), 126.2 (2 x $\underline{C}H_{ar}$), 132.4, 144.1, 146.6, 153.4, 154.9 (5 x $\underline{C}_{ar}$), 172.8 (C=O); m/z (ESi +ve, 45Kv) 337 (100%, $[MH]^+$); m/z (FAB, 3-NOBA) 337 (65%, $[MH]^+$), 307 (100%, $[MH\text{-}CH_2CH_3]^+$), 267 (30%, $[MH\text{-}COCH_2CH_2CH_3]^+$); Found (FAB) 337.17802 $C_{18}H_{21}N_6O_1$ requires 337.17768.

5.4.13 1-[5-(4-dimethylamino-2-methoxy-phenylazo)-benzotriazol-1-yl]-(*S*)-3-phenyl-butan-1-one (138A) and 1-[6-(4-Dimethylamino-2-methoxy-phenylazo)-benzotriazol-1-yl]-(*S*)-3-phenyl- butan-1-one (138B).



(*S*)-138A

+



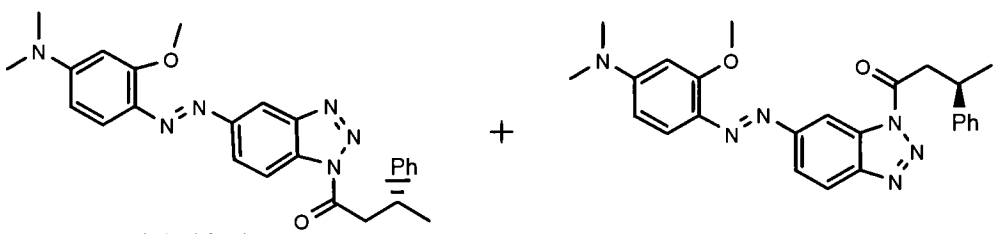
(*S*)-138B

The general procedure outlined in section **5.4.9** was followed with **122** (500mg, 1.69mmols) in 15ml of 'anhydrous' DCM. Triethylamine (285µl, 2.03mmols) and (*S*)-(+)-3-phenylbutyryl chloride (339mg, 1.86mmols) were added. The title compounds were furnished as a red solid (621.5mg, 84%) after trituration with CH_3CN. The ratio of the regioisomers was calculated by comparison of the integrals at 8.47 and 8.58 p.p.m and determined to be 44:56 (**A**:**B**) Separation of the regioisomers by HPLC or TLC was not achieved despite extensive method development.

R_f (DCM, 100%) 0.88 and 0.90 (DCM/EtOAc, 95/5) 0.40 and 0.38; R_t (87% CH_3CN) 7mins; Mp 159-161°C; υ_{max}(thin film)/cm^{-1} 3029 (CH_{ar}), 2964, 2926, 2871 (CH), 1742 (C=O), 1606 (C=C); δ_H ($CDCl_3$; 250MHz) 1.44 (1.5H, d, J 6.5Hz, C<u>H</u>$_3$, one regioisomer), 1.45 (1.5H, d, J 6.5Hz, C<u>H</u>$_3$, one regioisomer), 3.11 (3H, s, N(C<u>H</u>$_3$)$_2$, one regioisomer), 3.11(3H, s, N(C<u>H</u>$_3$)$_2$, one regioisomer), 3.61-3.69 (2H, m, C<u>H</u>$_2$CO), 3.74-3.82 (1H, m, C<u>H</u>CH$_2$CO, both regioisomers overlapping), 4.03 (1.5H, s, OC<u>H</u>$_3$, one regioisomer), 4.04 (1.5H, s, OC<u>H</u>$_3$, one regioisomer), 6.19 (0.5H, d, J 2.5Hz, C<u>H</u>$_{ar}$, one regioisomer), 6.21 (0.5H, d, J 2.5Hz, C<u>H</u>$_{ar}$, one regioisomer), 6.34 (0.5H, d, J 9.3 and 2.5Hz, C<u>H</u>$_{ar}$, one regioisomer), 6.35 (0.5H, d, J 9.3 and 2.5Hz, C<u>H</u>$_{ar}$, one regioisomer), 7.18-7.35 (5H, m, Ar<u>H</u>, both regioisomers), 7.83 (1H, dd, J 9.3 and 1.1Hz, C<u>H</u>$_{ar}$, both regioisomers), 8.00 (0.5H, dd, J 8.9 and 1.7Hz, CH_{bt}, **B**), 8.08 (0.5H, dd, J 8.9 and 0.8Hz, CH_{bt}, **B**), 8.18 (0.5H, dd, J 8.9 and 1.7Hz, CH_{bt}, **A**), 8.25 (0.5H, dd, J 8.9 and 0.8Hz, CH_{bt}, **A**), 8.47 (0.5H, dd, J 1.7 and 0.7Hz, CH_{bt}, **A**), 8.58 (0.5H, dd, J 1.7 and

0.7Hz, CH_{bt}, **B**); δ_c ($CDCl_3$; 63MHz) 21.9, 22.0 ($CH\underline{C}H_3$, both regioisomers), 35.9, 36.0 (<u>C</u>H, both regioisomers), 40.2 ($N(\underline{C}H_3)_2$), 43.3, 43.5 ($\underline{C}H_2CH$), 56.0, 56.1 ($O\underline{C}H_3$, both regioisomers), 94.4, 94.4 ($\underline{C}H_{ar}$, both regioisomers), 104.8, 104.9 ($\underline{C}H_{ar}$, both regioisomers), 108.8 ($\underline{C}H_{bt}$, **B**), 114.1 ($\underline{C}H_{bt}$, **A**), 114.2 ($\underline{C}H_{bt}$, **A**), 118.1, 118.3 ($\underline{C}H_{ar}$, both regioisomers), 119.9 ($\underline{C}H_{bt}$, **B**), 120.2 ($\underline{C}H_{bt}$, **B**), 124.5 ($\underline{C}H_{bt}$, **A**), 126.5 ($\underline{C}H_{ar}$, both regioisomers), 126.7 (2 x $\underline{C}H_{ar}$, both regioisomers), 128.5 (2 x $\underline{C}H_{ar}$, both regioisomers), 130.9, 131.8, 133.2, 133.5, 145.0, 145.0, 145.8, 146.9, 152.1, 154.4, 154.7, 155.1, 159.5, 159.9 (14 x $\underline{C}_{ar}$, both regioisomers), 170.9 and 170.9(C=O, both regioisomers); m/z (ESi +ve, 45kV) 443.2 (100%, $[MH]^+$); (FAB+, NOBA) 443 (100%, $[MH]^+$), 297 (30%, $[MH - COCH_2CHPhCH_3]^+$; (FAB+, NOBA) Found 443.21959 $C_{25}H_{26}N_6O_2$ requires 443.21955.

5.4.14 1-[5-(4-Dimethylamino-2-methoxy-phenylazo)-benzotriazol-1-yl]-(*R*)-3-phenyl-butan-1-one (138A) 1-[6-(4-Dimethylamino-2-methoxy-phenylazo)-benzotriazol-1-yl]-(*R*)-3-phenyl- butan-1-one (138B)

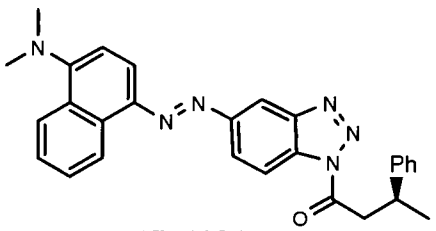


(*R*)-**138A** + (*R*)-**138B**

The general procedure (**5.4.9**) was followed with **122** (451mg, 1.52mmols) in 15ml of 'anhydrous' DCM. Triethylamine (258µl, 1.82mmols) and (*R*)-(-)-3-phenylbutyryl chloride (306mg, 1.67mmols) were added. The title compounds were furnished as a red solid (557mg, 83%) after trituration with CH_3CN. The ratio of the regioisomers was determined to be 55:45 (**B**:**A**) by comparison of the integrals at 8.51 and 8.63 p.p.m. Separation of the regioisomers, by HPLC or TLC, was not achieved despite extensive method development.

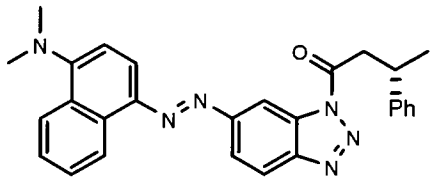
Analysis comparable to **5.4.13** except (FAB+, NOBA) Found 443.21963 $C_{25}H_{26}N_6O_2$ requires 443.21955.

5.4.15 1-[5-(4-Dimethylamino-napthalen-1-ylazo)-benzotriazol-1-yl]-(*S*)-3-phenyl-butan-1-one (139A) and 1-[6-(4-dimethyl-aminonapthalen-1-ylazo)-benzotriazol-1-yl]-(*S*)-3-phenyl-butan-1-one (139B)



(*S*)-139A

+



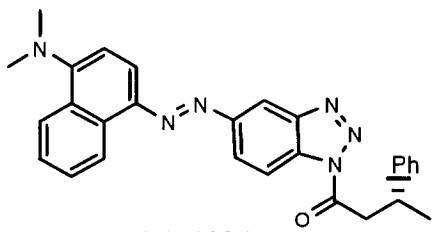
(*S*)-139B

The general procedure (**5.4.9**) was followed with **123** (500mg, 1.57mmols) in 15ml of 'anhydrous' DCM. Triethylamine (264µl, 1.88mmols) and (*S*)-3-phenylbutyryl chloride **106** (317mg, 1.73mmols) were added dropwise. The title compounds were furnished as a deep red oil (384mg, 57%) after column chromatography on silica gel eluting with DCM. The ratio of the regioisomers was determined to be 50:50 by comparison of the integrals at 8.67 and 8.79 p.p.m. Separation of the regioisomers by HPLC was not attempted.

R_f (DCM) 0.66 both regioisomers, υ_{max} (thin film)/cm^{-1} 3027 (CHar), 2941, 2871, 2836, 2788 (CH), 1739 (C=O), 1604 (C=C), 1572(C=C), 1509(C=C). δ_H ($CDCl_3$; 250MHz) 1.47(1.5H, d, J 6.4Hz, C<u>H</u>$_3$, one regioisomer), 1.48 (1.5H, d, J 6.4Hz, C<u>H</u>$_3$, one regiosiomer), 3.04(3H, s, N(C<u>H</u>$_3$)$_2$), one regioisomer), 3.05(3H, s, N(C<u>H</u>$_3$)$_2$, one regioisomer), 3.61-3.79 (2H, m, C<u>H</u>$_2$CO, both regioisomers overlapping), 3.80-3.90 (1H, m, C<u>H</u>CH$_2$CO, both regioisomers overlapping), 7.08 (0.5H, d, J 8.4Hz, C<u>H</u>$_{ar}$, one regioisomer), 7.09 (0.5H, d, J 8.4Hz, C<u>H</u>$_{ar}$, one regioisomer), 7.18-7.28 (5H, m, Ar<u>H</u>, both regioisomers), 7.57 (1H, ddd, J 8.5, 6.9 and 1.5Hz, C<u>H</u>$_{ar}$, both regioisomers), 7.66

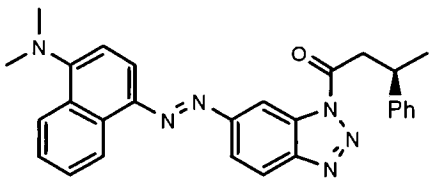
(1H, ddd, J 8.5, 6.9 and 1.5Hz, C$\underline{H}_{ar}$, both regioisomers), 7.94 (0.5H, d, J 8.4Hz, C$\underline{H}_{ar}$, one regioisomer), 7.95 (0.5H, d, J 8.4Hz, C$\underline{H}_{ar}$, one regioisomer), 8.19-8.35 (3H, m, 2 x C$\underline{H}_{bt}$ and C$\underline{H}_{ar}$, both regioisomers), 8.67 (0.5H, dd, J 1.3 and 1.3Hz, C$\underline{H}_{bt}$, **A**), 8.79 (0.5H, dd, J 1.3 and 1.3Hz, C$\underline{H}_{bt}$, **B**), 8.99-9.06 (1H, m, C$\underline{H}_{ar}$, both regioisomers); δ_c ($CDCl_3$; 63MHz) 22.0 ($\underline{C}H_3$, both regioisomers), 36.0 ($\underline{C}H$, both regioisomers), 43.5 ($\underline{C}H_2$, both regioisomers), 44.7 (N($\underline{C}H_3$)$_2$, both regioisomers), 109.9, 112.7, 112.9, 113.1, 113.7, 114.5, 115.2, 120.2, 120.4, 122.7, 123.6, 124.0, 124.6, 125.0, 125.3, 125.5 and 126.6 (17 x $\underline{C}H_{ar}$, both regioisomers), 126.2 (2 x $\underline{C}H_{ar}$), 127.0, 127.8 and 128.0 (3 x $\underline{C}H_{ar}$, both regioisomers), 128.2 ($\underline{C}_{ar}$), 128.5 (2 x $\underline{C}H_{ar}$), 131.6, 131.7, 132.0, 133.1, 133.3, 142.2, 142.4, 145.0, 145.0, 146.4, 146.8, 151.6, 154.3, 154.9, 155.3 (15 x C_{ar}, both regioisomers), 170.9 (C=O, both regioisomers); m/z (ESi +ve, 45kV) 463.2 (10%, $[MH]^+$); m/z (FAB, 3-NOBA) 463 (100%, $[MH]^+$), 317 (30%, $[MH\text{-}COCH_2CHPhCH_3]^+$), 77 (20%, Ph); (FAB, 3-NOBA) Found 463.22500, $C_{28}H_{26}N_6O$ requires 463.22463.

5.4.16 1-[5-(4-Dimethylamino-napthalen-1-ylazo)-benzotriazol-1-yl]-(*R*)-3-phenyl-butan-1-one (139A) and 1-[6-(4-dimethyl-aminonapthalen-1-ylazo)-benzotriazol-1-yl]-(*R*)-3-phenyl-butan-1-one (139B).



(*R*)-139A

+



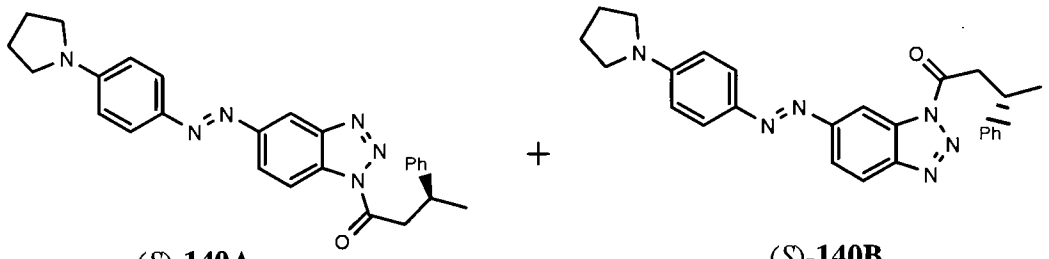
(*R*)-139B

The general procedure (**5.4.9**) was followed with **123** (337mg, 1.07mmols) in 15ml of 'anhydrous' DCM. Solutions of triethylamine (180µl, 1.28mmols) and (*R*)-3-phenylbutyryl chloride **106** (214mg, 1.17mmols) were added drop wise. The title compounds were furnished as a deep red oil (420mg, 85%) after column chromatography on silica gel eluting with DCM. The ratio of the regioisomers was

determined to be 50:50 by comparison of the integrals at 8.67 and 8.79 p.p.m. Separation of the regioisomers by HPLC was not attempted.

Analytical data comparable to (**5.4.15**) m/z (FAB, 3-NOBA) Found 463.22472, $C_{28}H_{26}N_6O$ requires 463.22463.

5.4.17 (*S*)-3-Phenyl-1-[6-(4-pyrollidin-1-yl-phenylazo)-benzotriazol-1-yl]-butan-1-one (140A) and (*S*)-3-Phenyl-1-[5-(4-pyrollidin-1-yl-phenylazo)-benzotriazol-1-yl]-butan-1-one (140B).



(*S*)-**140A** + (*S*)-**140B**

The general procedure (**5.4.9**) was followed with **124** (498mg, 1.79mmols) in 15ml of 'anhydrous' DCM. Solutions of triethylamine (302µl, 2.15mmols) and (*S*)-3-phenylbutyryl chloride **106** (362mg, 1.97mmols) were added drop wise. The title compounds were furnished as a bright red solid (373mg, 49%) after purification by column chromatography on silica gel eluting with DCM. The ratio of the regioisomers was determined to be 50:50 by comparison of the integrals at 8.48 and 8.60 p.p.m. Separation of the regioisomers by HPLC was not attempted.

R_f (DCM) 0.45 both regioisomers, υ_{max}(thin film)/cm^{-1} 3027 (CH_{ar}), 2923, 2836 (CH), 1736 (C=O), 1602 (C=C), 1518(C=C); δ_H ($CDCl_3$; 250MHz) 1.46 (3H, d, J 6.4Hz, C<u>H</u>$_3$, both regioisomers), 1.99-2.09 (4H, m, 2 x NCH$_2$C<u>H</u>$_2$, both regioisomers), 3.37-3.42 (2H, m, CHC<u>H</u>$_2$, both regioisomers), 3.69-3.83 (1H, m, C<u>H</u>CH$_2$, both regioisomers), 6.60 (2H, d, J 8.4Hz, 2 x C<u>H</u>$_{ar}$, both regioisomers), 7.16-7.35 (5H, m, Ar<u>H</u>), 7.90 (2H, d, 8.4Hz, 2 x C<u>H</u>$_{ar}$, both regioisomers), 8.02 (0.5H, dd, J 8.8 and 1.7Hz, C<u>H</u>$_{bt}$, **B**), 8.11

(0.5H, d, J 8.8, C$\underline{H}_{bt}$, **B**), 8.19 (0.5H, dd, J 8.8 and 1.7Hz, C$\underline{H}_{bt}$, **A**), 8.27 (0.5H, d, J 8.8, C$\underline{H}_{bt}$, **A**), 8.48 (0.5H, dd, J 1.7Hz, C$\underline{H}_{bt}$, **A**) and 8.60 (0.5H, d, J 1.7Hz, C$\underline{H}_{bt}$, **B**); δ_c ($CDCl_3$; 63MHz) 21.9 ($\underline{C}H_3$, both regioisomers), 25.3 (2 x $NCH_2\underline{C}H_2$, both regioisomers), 35.9 ($\underline{C}H$, both regioisomers), 43.4 ($CH\underline{C}H_2$, both regioisomers), 47.6 (2 x $N\underline{C}H_2CH_2$, both regioisomers), 108.0 ($\underline{C}H_{bt}$, **B**), 111.4 (2 x $\underline{C}H_{ar}$, one regioisomer), 111.4 (2 x $\underline{C}H_{ar}$, one regioisomer), 114.0, 114.2 (2 x $\underline{C}H_{bt}$, **A**), 120.0 ($\underline{C}H_{bt}$, **B**), 120.7 ($\underline{C}H_{bt}$, **B**), 124.3 ($\underline{C}H_{bt}$, **A**), 125.5 (2 x $\underline{C}H_{ar}$, one regioisomer), 125.8 ($\underline{C}H_{ar}$, both regioisomers), 126.5 ($\underline{C}H_{ar}$ x 2, one regioisomer), 126.7 ($\underline{C}H_{ar}$ x 2, both regioisomers), 128.5 ($\underline{C}H_{ar}$ x 2, both regioisomers), 131.0, 131.8, 142.9, 143.2, 145.0, 145.0, 145.9, 146.9, 150.3, 150.5, 151.6 and 154.5 (12 x $\underline{C}_{ar}$, both regioisomers), 170.8 (C=O); m/z (ESi+, 45kV) 439.3 (80%, $[MH]^+$); m/z (FAB, 3-NOBA), 439 (40%, $[MH]^+$) Found 439.22525 $C_{26}H_{26}N_6O$ requires 439.22463.

5.4.18 General procedure for preparation of ester 'blocked'SERRS dyes

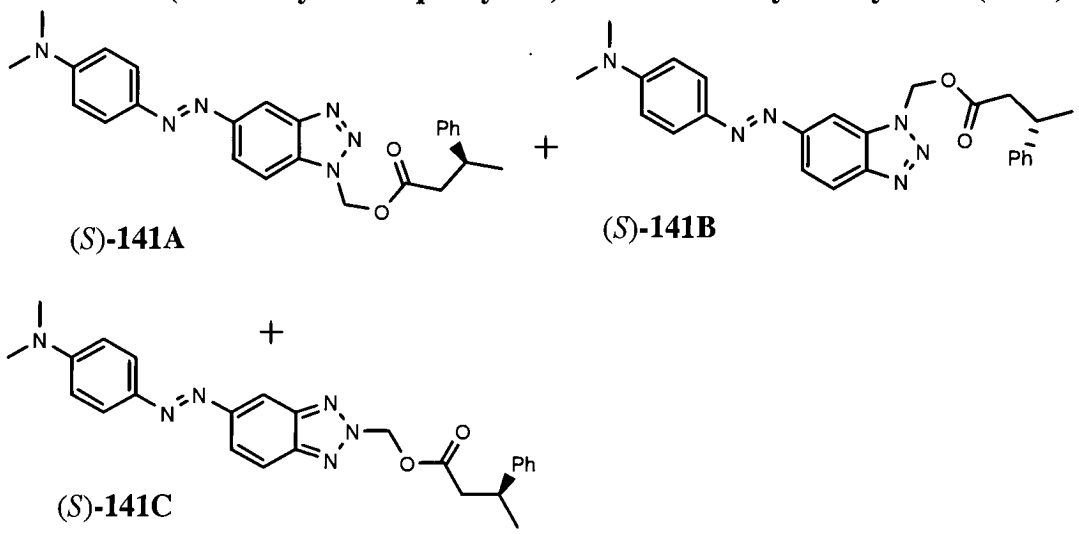
The acylated monoazo benzotriazole dyes **137-140(A+B)** were dissolved in the appropriate volume (depending on solubility) of CH_3CN:DCM (10:1) at room temperature and stirred vigorously. To this was added crushed K_2CO_3 (15 to 25 mol% - with respect to acylated monoazo benzotriazole dye) and formaldehyde (1.05 equiv, 37% aqueous solution). The resulting solution was stirred overnight at room temperature and monitored by TLC to ensure the reaction had gone to completion. The K_2CO_3 was removed by filtration, and then the solvent removed under reduced pressure to give the desired products as a highly coloured oil. The desired 'ester' compounds were obtained by column chromatography on silica gel. This provided the **C** ester as a single regioisomer and the **A** and **B** esters as a mixture of both regioisomers. A known weight of the **A** + **B** ester mixture was dissolved in the minimum volume of CH_3CN (with a few drops of DMSO added to aid solubility) to give a typical concentration of 5-10mg/ml. The mixture was purified by prep-HPLC on a Biotage Flex system eluting with the appropriate mobile phase. The individual fractions of each regioisomer were pooled, the organic solvent removed under reduced pressure and the ester compound isolated from

the remaining aqueous solution by extraction with DCM. The organic layer was separated, dried over $MgSO_4$, filtered and evaporated under reduced pressure to yield a single regioisomer of the ester compound as a highly coloured oil. This oil was re-purified by small-scale column chromatography on silica gel (to remove any underivatised monoazo dye which may have been carried through the purification procedure) to yield the desired ester product as a highly coloured oil.

Note: The chiral integrity of the ester compounds was established by cleavage of ≈1mg dissolved in 2ml of 1M NaOH / acetone (50:50). The solvent was removed under reduced pressure and the residue resuspended in IPA / Hexane (50:50), filtered through a 0.22μm filter and the resulting solution analysed by normal phase chiral HPLC using a Chiracel OD column eluting with Hexane / IPA (98:2, 0.1%TFA) at 5°C.
R_t of (*R*)-3 phenylbutyric acid = 8.0mins
R_t of (*S*)-3 phenylbutyric acid = 17.8mins

The enantiomeric excess determined was compared with that for the starting 3-phenylbutyric acid as determined by the same method.
Starting (*S*)-3-Phenylbutyric acid = >99% e.e.
Starting (*R*)-3-Phenylbutyric acid = 100% e.e.

5.4.19 (*S*)-3-Phenyl-butyric acid 5-(4-dimethyamino-phenylazo)-benzotriazol-1-ylmethyl ester (141A) and (*S*)-3-Phenyl-butyric acid 6-(4-dimethylamino-phenylazo)-benzotriazol-1-ylmethyl ester (141B) and (*S*)-3-Phenyl-butyric acid 5-(4-dimethylamino-phenylazo)-benzotriazol-2-yl methyl ester (141C).



(*S*)-**141A** (*S*)-**141B**

(*S*)-**141C**

The general protocol outlined in section **5.4.18** was followed with (*S*)-**137A+B** (55.4mg, 0.134mmols) dissolved in 10ml of CH_3CN:DCM (9:1). Crushed K_2CO_3 (4.6mg, 0.033mmols, 25 mol %) and formaldehyde (10.5µl, 0.141mmols, 1.05 equiv.) were added and the reaction stirred vigorously overnight at room temperature. Column chromatography eluting with DCM, furnished the desired products as dark red oils; **A+B**(19.8mg, 33%) as a mixture of the regioisomers in the ratio (52 **B**: 48 **A**) and **C** (1.9mg, 3.2%). **A+B** were separated using the Biotage Flex system running a gradient from 75:25 to 95:5 over 20mins (CH_3CN:H_2O) to give **141B** as a red oil (7.0mg, 11.7%) and **141A** as a red oil (6.6mg, 11%).

(*S*)-**141A**

R_f (DCM) 0.16; R_t 11.6mins; υ_{max}(thin film)/cm^{-1} 3054 (CH_{ar}), 2963 (CH), 1753 (C=O), 1602 (C=C) and 1519 (C=C); δ_H ($CDCl_3$; 600MHz) 1.26 (3H, d, J 7.1Hz, $C\underline{H}_3$), 2.66 (1H, dd, J 15.2 and 7.6Hz, $C\underline{H}_aH_bCO$), 2.69 (1H, dd, J 15.2 and 7.8Hz, $CH_a\underline{H}_bCO$), 3.15 (6H, s, $N(CH_3)_2$), 3.26 (1H, m, $C\underline{H}$), 6.53 (1H, d, J 11.1Hz, $C\underline{H}_aH_bO$), 6.56 (1H, d, J

11.2Hz, $CH_a\underline{H}_bO$), 6.82 (2H, d, J 9.2Hz, $C\underline{H}_{ar}$ x 2), 7.07-7.18 (5H, m, Ar$\underline{H}$), 7.64 (1H, dd, J 8.9 and 0.6Hz, $C\underline{H}_{bt}$) 7.96 (2H, d, J 9.3Hz, $C\underline{H}_{ar}$ x 2), 8.14 (1H, dd, J 8.9 and 1.6Hz, $C\underline{H}_{bt}$) and 8.52 (1H, dd, J 1.6 and 0.6Hz, $C\underline{H}_{bt}$); ^{1}H NOE ($CDCl_3$; 360MHz) irradiation at 7.64 p.p.m gave 2% enhancement at 6.53-6.56 p.p.m and 17% enhancement at 8.14 p.p.m and irradiation at 6.56-6.59 p.p.m gave 10% enhancement at 7.64 p.p.m); δ_c ($CDCl_3$; 63MHz) 22.7 ($\underline{C}H_3$), 36.5 ($\underline{C}H$), 40.3 ($N(\underline{C}H_3)_2$), 42.3 ($\underline{C}H_2CO$), 67.6 (CH_2O), 110.1 ($\underline{C}H_{bt}$), 111.5 ($\underline{C}H_{ar}$ x 2), 115.3 ($\underline{C}H_{bt}$), 125.2 ($\underline{C}H_{ar}$ x 2), 125.3 ($\underline{C}H_{bt}$), 126.4 ($\underline{C}H_{ar}$ x 2), 126.6 ($\underline{C}H_{ar}$), 128.4 ($\underline{C}H_{ar}$ x 2), 133.1, 143.4, 144.5, 146.8, 150.7, 152.6 ($\underline{C}_{ar}$ x 6) and 171.4 (C=O); m/z (ESi+, 45kV) 443.4 (100%, $[MH]^+$), 267 (50%, $[MH - CH_2OCOCH_2CHPhCH_3]^+$); m/z (FAB, NOBA) 443 (100%, $[MH]^+$), 289 (50%, $[MNa - CH_2OCOCH_2CHPhCH_3]^+$), 267 (30%, $[MH - CH_2OCOCH_2CHPhCH_3]^+$); (FAB, NOBA) Found 443.21950 $C_{25}H_{27}N_6O_2$ requires 443.21955.

(*S*)-**141B**

R_f (DCM) 0.16; R_t 11.3mins; υ_{max}(thin film)/cm^{-1} 3053 (CH_{ar}), 2963 (CH), 1752 (C=O), 1602 (C=C), 1519 (C=C); δ_H ($CDCl_3$; 600MHz) 1.27 (3H, d, J 7Hz, $C\underline{H}_3$), 2.66 (1H, dd, J 15.2 and 7.6Hz, $C\underline{H}_aH_bCO$), 2.71 (1H, dd, J 15.2 and 7.8Hz, $CH_a\underline{H}_bCO$), 3.16 (6H, s, $N(CH_3)_2$), 3.26 (1H, m, C$\underline{H}$), 6.56 (1H, d, J 11.2Hz, $C\underline{H}_aH_bO$), 6.59 (1H, d, J 11.2Hz, $CH_a\underline{H}_bO$), 6.82 (2H, d, J 9.3Hz, 2 x $C\underline{H}_{ar}$), 7.01-7.16 (5H, m, Ar$\underline{H}$), 7.98 (2H, d, J 9.3Hz, 2 x $C\underline{H}_{ar}$), 8.02 (1H, dd, J 9.3 and 1.6Hz, $C\underline{H}_{bt}$) overlapping with 8.03 (1H, d, 1.6Hz, $C\underline{H}_{bt}$) and 8.12 (1H, d, J 9.3Hz, $C\underline{H}_{bt}$); ^{1}H NOE ($CDCl_3$; 360MHz) irradiation at 8.03 p.p.m gave 2% enhancement at 6.56-6.59 p.p.m and irradiation at 6.56-6.59 p.p.m gave 7% enhancement at 8.03 p.p.m; δ_c ($CDCl_3$; 63MHz) 21.9 ($\underline{C}H_3$), 36.4 ($\underline{C}H$), 40.3 ($N(\underline{C}H_3)_2$), 42.4 ($\underline{C}H_2CO$), 67.8 ($\underline{C}H_2O$), 104.2 ($\underline{C}H_{bt}$), 111.5 ($\underline{C}H_{ar}$ x 2), 119.1 ($\underline{C}H_{bt}$), 120.1 ($\underline{C}H_{bt}$), 125.5 ($\underline{C}H_{ar}$ x 2), 126.5 ($\underline{C}H_{ar}$ x 2), 126.5 ($\underline{C}H_{ar}$), 128.4 ($\underline{C}H_{ar}$ x 2), 133.4, 143.5, 144.6, 146.2, 152.9, 153.1 ($\underline{C}_{ar}$ x 6) and 171.2 (C=O); m/z (ESi+, 45kV) 443.4 (100%, $[MH]^+$), 267 (50%, $[MH - CH_2OCOCH_2CHPhCH_3]^+$); m/z (FAB, NOBA) 443 (100%, $[MH]^+$), 289 (50%, $[MNa - CH_2OCOCH_2CHPhCH_3]^+$), 267 (30%, [MH –

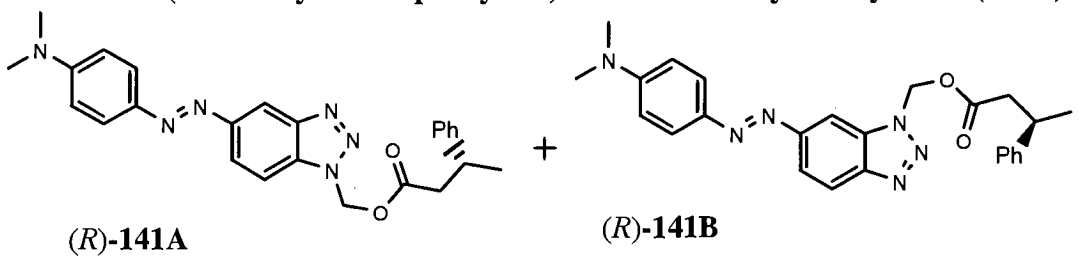
$CH_2OCOCH_2CHPhCH_3]^+$); (FAB, NOBA) Found 443.21947 $C_{25}H_{27}N_6O_2$ requires 443.21955.

(*S*)-**141C**

R_f (DCM) 0.41; υ_{max}(thin film)/cm^{-1} 3054 (CH_{ar}), 2987 (CH), 1757 (C=O), 1602 (C=C); δ_H ($CDCl_3$; 360MHz) 1.32 (3H, d, J 7.1Hz, $C\underline{H}_3$), 2.72 (1H, dd, J 15.4 and 7.8Hz, $C\underline{H}_aH_bCO$), 2.76 (1H, dd, J 15.4 and 7.4Hz, $CH_a\underline{H}_bCO$), 3.15 (6H, s, $N(CH_3)_2$), 3.33 (1H, m, C<u>H</u>), 6.53 (1H, d, J 10.0Hz, $C\underline{H}_aH_bO$), 6.56 (1H, d, J 10.0Hz, $CH_a\underline{H}_bO$), 6.82 (2H, d, J 9.2Hz, $C\underline{H}_{ar}$ x 2), 7.02-7.25 (5H, m, Ar<u>H</u>), 7.93 (1H, dd, J 9.3 and 0.7Hz, $C\underline{H}_{bt}$) 7.97 (2H, d, J 9.3Hz, $C\underline{H}_{ar}$ x 2), 8.14 (1H, dd, J 9.3 and 1.7Hz, $C\underline{H}_{bt}$) and 8.35 (1H, dd, J 1.7 and 0.7Hz, $C\underline{H}_{bt}$); ^1H NOE ($CDCl_3$; 360MHz) irradiation at 6.56 p.p.m gave no enhancement at any of the benzotriazole signals; δ_c ($CDCl_3$; 63MHz) 22.2 ($\underline{C}H_3$), 36.7 ($\underline{C}H$), 40.7 ($N(\underline{C}H_3)_2$), 42.8 ($\underline{C}H_2CO$), 75.4 ($\underline{C}H_2O$), 111.9 ($\underline{C}H_{ar}$ x 2), 116.1 ($\underline{C}H_{bt}$), 119.3 ($\underline{C}H_{bt}$), 121.3 ($\underline{C}H_{bt}$), 126.9 ($\underline{C}H_{ar}$), 127.0 ($\underline{C}H_{ar}$ x 2), 127.0 ($\underline{C}H_{ar}$ x 2), 128.9 ($\underline{C}H_{ar}$ x 2), 143.9, 145.3, 146.0, 146.1, 152.9, 153.1 ($\underline{C}_{ar}$ x 6) and 171.0 (C=O); m/z (ESi+, 45kV) 443.4 (100%, $[MH]^+$), 267 (50%, $[MH - CH_2OCOCH_2CHPhCH_3]^+$); m/z (FAB, NOBA) 443 (100%, $[MH]^+$), 289 (50%, $[MNa - CH_2OCOCH_2CHPhCH_3]^+$), 267 (30%, $[MH - CH_2OCOCH_2CHPhCH_3]^+$); (FAB, NOBA) Found 443.21959 $C_{25}H_{27}N_6O_2$ requires 443.21955.

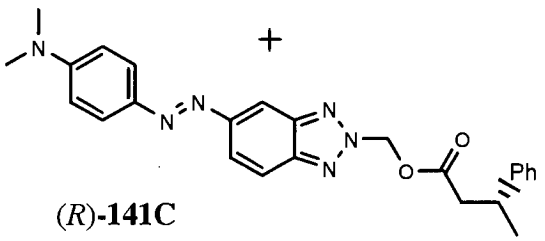
A, **B** and **C** were all cleaved and the resulting (*S*)–3 phenylbutyric acid released was determined by chiral HPLC (according to protocol **5.4.18**) to be >99% e.e. This is in excellent agreement with the starting acid.

5.4.20 (*R*)-3-Phenyl-butyric acid 5-(4-dimethyamino-phenylazo)-benzotriazol-1-ylmethyl ester (141A), (*R*)-3-Phenyl-butyric acid 6-(4-dimethylamino-phenylazo)-benzotriazol-1-ylmethyl ester (141B) and (*R*)-3-Phenyl-butyric acid 5-(4-dimethylamino-phenylazo)-benzotriazol-2-yl methyl ester (141C).



(*R*)-**141A**

(*R*)-**141B**



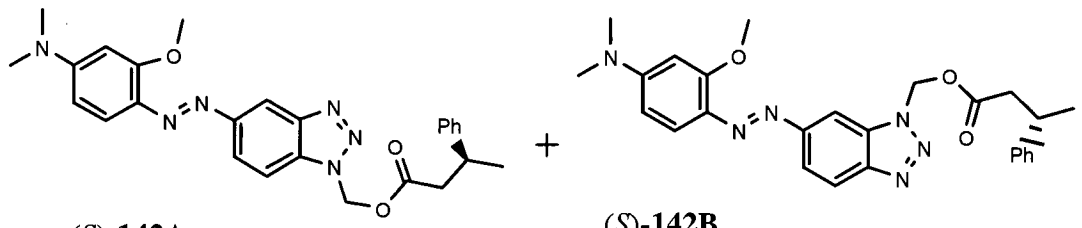
(*R*)-**141C**

The general protocol outlined in section **5.4.18** was followed with (*R*)-**137A+B** (108.3mg, 0.262mmols) dissolved in 20ml of CH_3CN:DCM (9:1). Crushed K_2CO_3 (9.02mg, 0.065mmols, 25 mol %) and formaldehyde (39.3µl, 0.524mmols, 2 equiv.) were added and the reaction stirred vigorously overnight at room temperature. Column chromatography eluting with DCM, furnished the desired products as dark red oils; **A+B**(35.7mg, 31%) as a mixture of the regioisomers in the ratio 50:50 as determined by NMR and **C** (3.4mg, 2.9%). **A+B** were separated using the Biotage Flex system running a gradient from 75:25 to 95:5 over 20mins (CH_3CN:H_20) to give **141B** as a red oil (9.7mg, 8.4%) and **141A** as a red oil (8.8mg, 7.6%).

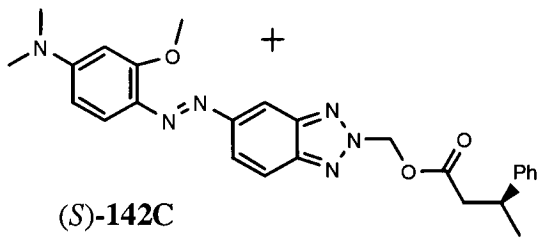
Analytical data for (*R*)-**141A**, (*R*)-**141B** and (*R*)-**141C** were all comparable to the appropriate (*S*)-enantiomers in **5.2.19.**

A, B and **C** were all cleaved and the resulting (*R*) – 3 phenylbutyric acid was determined according to protocol **5.4.18** to be >99% e.e. – which is in excellent agreement with the starting acid.

5.4.21 (*S*)-3-Phenyl-butyric acid 5-(4-dimethylamino-2-methoxy-phenylazo)-benzotriazol-1-yl methyl ester (142A), (*S*)-3-phenyl-butyric acid 6-(4-dimethylamino-2-methoxy-phenylazo)-benzotriazol-1-ylmethyl ester (142B) and (*S*)-3-phenyl-butyric acid 5-(4-dimethylamino-2-methoxy-phenylazo)-benzotriazol-2yl methyl ester (142C).



(*S*)-**142A** (*S*)-**142B**



(*S*)-**142C**

The general protocol **5.4.18** was followed with (*S*)-**138 A+B** (56.9mg, 0.129mmols) dissolved in 10ml of CH_3CN: DCM (9:1). Crushed K_2CO_3 (3.6mg, 0.035mols, 15 mol %) and formaldehyde (19.3µl, 0.135mmols, 1.05 equiv.) were added and the reaction stirred vigorously overnight at room temperature. Column chromatography eluting with DCM, furnished the desired products as 2 dark red oils; **A+B** (30.5mg, 50.8%) as a mixture of the regioisomers in the ratio 56:44 (ratio determined by integral of methyl peaks at 1.25 and 1.27 p.p.m) and **C** (4.6mg, 7.5%). **A+B** could not be separated by prep scale HPLC despite extensive method development.

142A+B

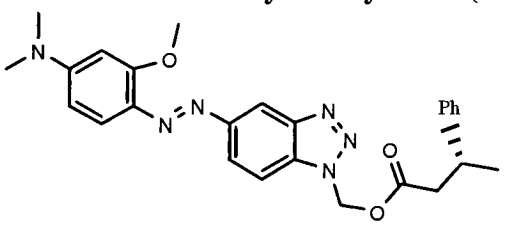
R_f (DCM/EtOAc, 9:1) 0.44 and 0.44; υ_{max}(thin film)/cm^{-1} 3053, 3029 (CH_{ar}), 2962, 2926 (CH), 1750 (C=O), 1602 (C=C) and 1559; δ_H ($CDCl_3$; 250MHz) 1.25 (1.5H, d, J 7.0Hz, C$\underline{H}_3$, one regioisomer), 1.27 (1.5H, d, J 7.0Hz, C$\underline{H}_3$, one regioisomer), 2.67 (1H, dd, J 15.2 and 7.7Hz, C$\underline{H}_a$H$_b$CO, both regioisomers), 2.68 (1H, dd, J 15.2 and 7.7Hz, C$\underline{H}_a$H$_b$CO, both regioisomers), 3.15 (3H, s, N(C$\underline{H}_3$)$_2$, one regioisomer), 3.17 (3H, s, N(C$\underline{H}_3$)$_2$, one regioisomer), 3.23-3.28 (1H, m, C$\underline{H}$, both regioisomers), 4.09 (3H, s, OC$\underline{H}_3$, both regioisomers), 6.25 (0.5H, d, J 2.6Hz, C$\underline{H}_{ar}$, one regioisomer), 6.26 (0.5H, d, J 2.6Hz, C$\underline{H}_{ar}$, one regioisomer), 6.41 (0.5H, dd, J 9.3 and 2.6Hz, C$\underline{H}_{ar}$, one regioisomer), 6.41 (0.5H, dd, J 9.3 and 2.6Hz, C$\underline{H}_{ar}$, one regioisomer), 6.41 (0.5H, dd, J 9.3 and 2.6Hz, C$\underline{H}_{ar}$, one regioisomer), 6.50 (0.5H, d, J 11.2Hz, C$\underline{H}_a$H$_b$O, one regioisomer), 6.52 (0.5H, d, J 11.2Hz, C$\underline{H}_a$H$_b$O, one regioisomer), 6.56 (0.5H, d, J 11.2Hz, CH$_a\underline{H}_b$O, one regioisomer), 6.59 (0.5H, d, J 11.2Hz, CH$_a\underline{H}_b$O, one regioisomer), 7.06-7.20 (5H, m, Ar$\underline{H}$, both regioisomers), 7.63 (0.5H, d, J 8.9Hz, C$\underline{H}_{bt}$, **A**), 7.91 (0.5H, d, J 9.3Hz, C$\underline{H}_{ar}$, one regioisomer), 7.92 (0.5H, d, J 9.3Hz, C$\underline{H}_{ar}$, one regioisomer), 7.99-8.03 (1H, m, overlapping signals from 2 x C$\underline{H}_{bt}$), 8.09 (0.5H, dd, J 8.6 and 1.0Hz, C$\underline{H}_{bt}$, **B**), 8.14 (0.5H, dd, J 9 and 1.6Hz, C$\underline{H}_{bt}$, **A**) and 8.50 (0.5H, dd, J 1.6 and 1.0Hz, C$\underline{H}_{bt}$, **A**); δ_c ($CDCl_3$; 63MHz) 22.3 (C$\underline{H}_3$, both regioisomers), 36.8 (C$\underline{H}$, both regioisomers), 40.8 (N(C$\underline{H}_3$)$_2$), 42.8 (C$\underline{H}_2$CO), 56.6 (OC$\underline{H}_3$, both regioisomers), 68.0, 68.4 (C$\underline{H}_2$O, both regioisomers), 94.9, 95.1 (C$\underline{H}_{ar}$, both regioisomers) 104.2 (C$\underline{H}_{bt}$, **B**) 105.7 (C$\underline{H}_{ar}$, both regioisomers), 110.5 (C$\underline{H}_{bt}$, **A**), 115.3 (C$\underline{H}_{bt}$, **A**), 118.9, 119.2 (C$\underline{H}_{ar}$, both regioisomers), 119.8 (C$\underline{H}_{bt}$, **B**), 120.5 (C$\underline{H}_{bt}$, **B**), 122.8 (C$\underline{H}_{bt}$, **A**), 126.4 (C$\underline{H}_{ar}$, both regioisomers) 126.9 (C$\underline{H}_{ar}$ x 2, both regioisomers), 128.9 (C$\underline{H}_{ar}$ x 2, both regioisomers), 132.7, 133.0, 133.3, 133.4, 144.5, 145.8, 146.6, 150.8, 153.2, 154.4, 154.7, 159.4 and 159.8 (C$\underline{}_{ar}$ x 13), 171.0 and 171.2 (C=O, both regioisomers); m/z (ESi+, 45kV) 473.2 (100%, $[MH]^+$), 297 (10%, $[MH - CH_2OCOCH_2CHPhCH_3]^+$); m/z (FAB, NOBA) 473 (100%, $[MH]^+$), 297 (30%, $[MH - CH_2OCOCH_2CHPhCH_3]^+$); (FAB, THIO) Found 473.23011 $C_{26}H_{28}N_6O_3$ requires 473.23011.

142C

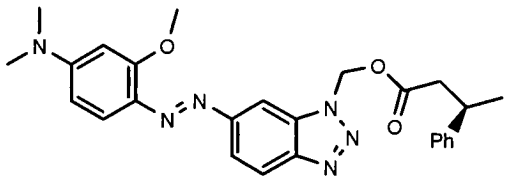
R_f (DCM/EtOAc, 9:1) 0.63; υ_{max}(thin film)/cm^{-1} 3053, 3029 (CH_{ar}), 2962, 2926 (CH), 1756 (C=O), 1602 (C=C) and 1559; δ_H ($CDCl_3$; 250MHz) 1.31 (3H, d, J 7.0Hz, C$\underline{H}_3$), 2.71 (1H, dd, J 15.4 and 7.8Hz, C$\underline{H}_a$H$_b$CO), 2.74 (1H, dd, J 15.4 and 7.6Hz, C$\underline{H}_a$H$_b$CO), 3.16 (6H, s, N(CH_3)$_2$), 3.28-3.36 (1H, m, C$\underline{H}$,), 4.09 (3H, s, OC$\underline{H}_3$), 6.27 (1H, d, J 2.6Hz, C$\underline{H}_{ar}$), 6.40 (1H, dd, J 9.2 and 2.6Hz, C$\underline{H}_{ar}$), 6.51 (1H, d, J 9.9Hz, C$\underline{H}_a$H$_b$O), 6.51 (1H, d, J 9.9Hz, C$\underline{H}_a$H$_b$O), 7.11-7.25 (5H, m, Ar$\underline{H}$), 7.90 (0.5H, d, J 9.2Hz, C$\underline{H}_{ar}$), 7.90 (1H, dd, J 9.4 and 0.8Hz, C$\underline{H}_{bt}$), 8.13 (1H, dd, J 9.3 and 1.7Hz, C$\underline{H}_{bt}$) and 8.30 (1H, dd, J 1.6 and 0.8Hz, C$\underline{H}_{bt}$); δ_c ($CDCl_3$; 63MHz) 21.7 ($\underline{C}H_3$), 36.2 ($\underline{C}$H), 40.3 (N($\underline{C}H_3$)$_2$), 42.2 ($\underline{C}H_2$), 56.2 (O$\underline{C}H_3$), 74.9 ($\underline{C}H_2$O), 94.6 ($\underline{C}H_{ar}$), 104.9 ($\underline{C}H_{ar}$), 115.0 ($\underline{C}H_{bt}$), 118.1 ($\underline{C}H_{ar}$), 118.7 ($\underline{C}H_{bt}$), 121.3 ($\underline{C}H_{bt}$), 126.4 ($\underline{C}H_{ar}$), 126.5 (2 x $\underline{C}H_{ar}$), 128.4 (2 x $\underline{C}H_{ar}$), 133.3, 144.8, 145.4, 145.5, 152.9, 154.4, 159.7 (7 x $\underline{C}_{ar}$) and 170.5 (C=O); m/z (ESi+, 45kV) 473.2 (100%, $[MH]^+$), 297 (25%, $[MH - CH_2OCOCH_2CHPhCH_3]^+$); m/z (FAB, 3-NOBA) 473 (100%, $[MH]^+$), 297 (30%, $[MH - CH_2OCOCH_2CHPhCH_3]^+$); (FAB, 3-NOBA) Found 473.23057 $C_{26}H_{28}N_6O_3$ requires 473.23011.

A, **B** and **C** were all cleaved and the resulting (*S*)–3 phenylbutyric acid released was determined by chiral HPLC (according to protocol **5.4.18**) to be >99% e.e. This is in excellent agreement with the starting acid.

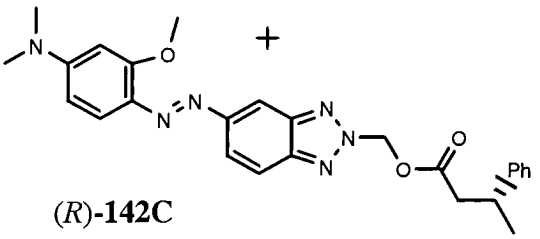
5.4.22 (*R*)-3-Phenyl-butyric acid 5-(4-dimethylamino-2-methoxy-phenylazo)-benzotriazol-1-yl methyl ester (142A), (*R*)-3-phenyl-butyric acid 6-(4-dimethylamino-2-methoxy-phenylazo)-benzotriazol-1-ylmethyl ester (142B) and (*R*)-3-phenyl-butyric acid 5-(4-dimethylamino-2-methoxy-phenylazo)-benzotriazol-2yl methyl ester (142C).



(*R*)-**142A**



(*R*)-**142B**



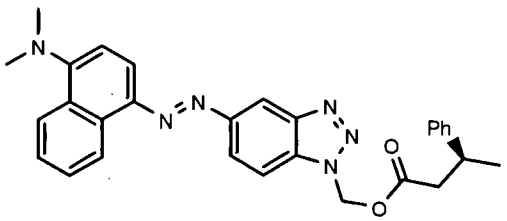
(*R*)-**142C**

The general protocol outlined in section **5.4.18** was followed with (*R*)-**138(A+B)** (102.8mg, 0.232mmols) dissolved in 15ml of CH_3CN: DCM (9:1). Crushed K_2CO_3 (4.8mg, 0.035mols, 15 mol %) and formaldehyde (35.3µl, 0.244mmols, 1.05 equiv.) were added and the reaction stirred vigorously overnight at room temperature. Column chromatography eluting with DCM, furnished the desired products as dark red oils; **A+B** (52.9mg, 48%) as a mixture of the regioisomers in the ratio 55:45 (as determined by integral of methyl peaks at 1.25 and 1.27 p.p.m) and **C** (4.6mg, 4.2%). **A+B** could not be separated by prep scale HPLC despite extensive method development.

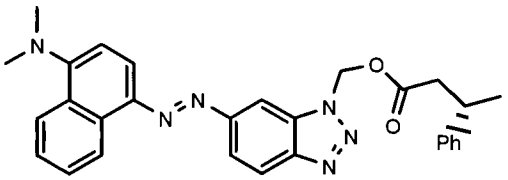
Analytical data for (*R*)-**142A**, (*R*)-**142B** and (*R*)-**142C** were all comparable to the appropriate (*S*)-enantiomers in **5.4.21.**

A, **B** and **C** were all cleaved and the resulting (*R*)–3 phenylbutyric acid released determined, according to protocol **5.4.18,** to be >99% e.e. – which is in excellent agreement with starting acid.

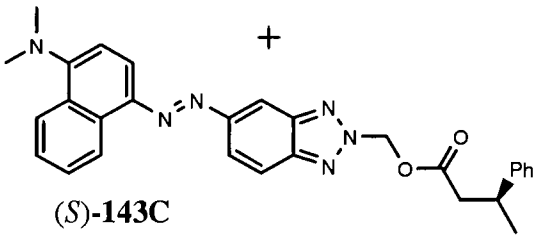
5.4.23 (*S*)-3-Phenyl-butyric acid 5-(4-dimethylamino-napthalen-1-ylazo)-benzotriazol-1-ylmethyl ester (143A), (*S*)-3-Phenyl-butyric acid 6-(4-dimethylamino-napthalen-1-ylazo)-benzotriazol-1-yl methyl ester (143B) and (*S*)-3-Phenyl-butyric acid 5-(4-dimethylamino-napthalen-1-ylazo)-benzotriazol-2-yl methyl ester (143C).



(*S*)-**143A**



(*S*)-**143B**



(*S*)-**143C**

The general protocol (**5.4.18**) was followed with (*S*)-**139A+B** (241.9mg, 0.524mmols) dissolved in 40ml of CH_3CN: DCM (9:1). Crushed K_2CO_3 (10.8mg, 0.0786mmols, 15 mol%) and formaldehyde (42.5μl, 0.549mmols, 1.05 equiv.) were added and the reaction stirred vigorously overnight at room temperature. Column chromatography eluting with DCM, furnished the desired products as dark red oils; **A+B** (212.2mg, 82%) as a mixture of both regioisomers in the ratio 48:52 (as determined by integral of peaks at 2.96, 6.45 and 9.0p.p.m) and **C** (19.7 mg, 7.6%). **A+B** (72.5mg) were taken and separated on a Biotage Flex system eluting at 50:50 (CH_3CN:H_2O) to provide **B** as a deep red oil (12.1mg) and **A** as an orange/red oil (25mg).

(*S*)-**143A**

R_f (DCM/Et_2O, 99:1) 0.31; R_t 25-29 mins (broad peak); υ_{max} (thin film)/cm^{-1} 3024 (CH_{ar}), 2964, 2924, 2825 (CH), 1750 (C=O), 1603 (C=C), 1574 (C=C), 1509 (C=C) 1393 and 1136; δ_H ($CDCl_3$; 600MHz, COSY) 1.24 (3H, d, J 7Hz, $C\underline{H}_3$), 2.66 (1H, dd, J 15.2 and 7.7Hz, $C\underline{H}_aH_bCO$), 2.67 (1H, dd, J 15.2 and 7.7Hz, $CH_a\underline{H}_bCO$), 3.20–3.29 (1H,

m, C<u>H</u>), 6.50 (1H, d, J 11.1Hz, C<u>H</u>$_a$H$_b$O), 6.56 (1H, d, J 11.1Hz, CH$_a$<u>H</u>$_b$O), 7.02-7.14 (6H, m, Ar<u>H</u> (x5) + C<u>H</u>$_{ar}$ naphthalene, overlapping), 7.58 (1H, ddd, J 8.4, 6.9 and 1.5Hz, C<u>H</u>$_{ar}$), 7.65 (1H, dd, J 8.9 and 0.7Hz, C<u>H</u>$_{bt}$) overlapping with 7.68 (1H, ddd, J 8.4, 6.9 and 1.5Hz, C<u>H</u>$_{ar}$), 7.95 (1H, d, J 8.3Hz, C<u>H</u>$_{ar}$), 8.23 (1H, ddd, J 8.3, 1.5 and 0.9Hz, C<u>H</u>$_{ar}$), 8.25 (1H, dd, J 8.9 and 1.7Hz, C<u>H</u>$_{bt}$), 8.65 (1H, dd, J 1.7 and 0.7Hz, C<u>H</u>$_{bt}$) and 9.05 (1H, ddd, J 8.4, 1.5 and 0.9Hz, C<u>H</u>$_{ar}$); ^{1}H NOE irradiation at 6.50-6.56 p.p.m gave 3.5% enhancement at 7.7p.p.m.; δ_c (CDCl$_3$; 63MHz) 21.9 (<u>C</u>H$_3$), 36.4 (<u>C</u>H), 42.2 (<u>C</u>H$_2$CO), 44.8 (2 x N(<u>C</u>H$_3$)$_2$), 67.4 (<u>C</u>H$_2$O), 110.2 (<u>C</u>H$_{bt}$), 112.5 (<u>C</u>H$_{ar}$), 113.1 (<u>C</u>H$_{ar}$), 116.3 (<u>C</u>H$_{bt}$), 123.6, 123.6, 124.5 (<u>C</u>H$_{ar}$ x 3), 125.3 (<u>C</u>H$_{bt}$), 126.3 (<u>C</u>H$_{ar}$ x 2), 126.4, 126.8 (<u>C</u>H$_{ar}$), 128.1 (<u>C</u>$_{ar}$), 128.3 (<u>C</u>H$_{ar}$ x 2), 133.1, 133.4, 142.4, 144.3, 146.6, 150.8, 154.7 (<u>C</u>$_{ar}$ x 7) and 171.3 (C=O); m/z (ESi+, 45kV) 493.2 (100%, [MH]$^+$); m/z (FAB, 3-NOBA) 493 (80%, [MH]$^+$), (FAB, 3-NOBA) Found 493.23554 $C_{29}H_{28}N_6O_2$ requires 493.23519.

(*S*)-**143B**

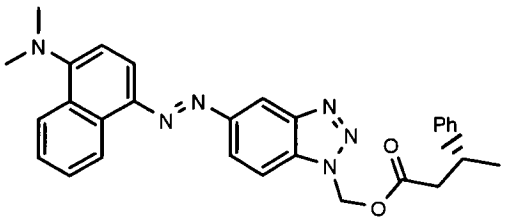
R_f (DCM/Et$_2$O, 99:1) 0.31; R_t 20-25mins (broad peak); υ_{max} (thin film)/cm^{-1} 3053, 3024 (CH$_{ar}$), 2964, 2922, 2825 (CH), 1752 (C=O), 1603 (C=C), 1573 (C=C), 1509(C=C) 1393 and 1136; δ_H CDCl$_3$; 250MHz) 1.24 (3H, d, J 7Hz, C<u>H</u>$_3$), 2.63 (1H, dd, J 15.4 and 7.7Hz, C<u>H</u>$_a$H$_b$CO), 2.68 (1H, dd, J 15.4 and 7.8Hz, CH$_a$<u>H</u>$_b$CO), 3.26 (1H, m, C<u>H</u>), 6.55 (1H, d, J 11.1Hz, C<u>H</u>$_a$H$_b$O), 6.61 (1H, d, J 11.1Hz, CH$_a$<u>H</u>$_b$O), 6.99-7.19 (6H, m, Ar<u>H</u> (x5) + C<u>H</u>$_{ar}$ naphthalene, overlapping), 7.58 (1H, ddd, J 8.4,6.9 and 0.7Hz, C<u>H</u>$_{ar}$) 7.68 (1H, ddd, J 8.3,6.9 and 1.5Hz, C<u>H</u>$_{ar}$), 7.97 (1H, d, J 8.4Hz , C<u>H</u>$_{ar}$) 8.14-8.17 (3H, m, C<u>H</u>$_{bt}$ x 3 overlapping), 8.24(1H, dd, J 8.1 and 0.9Hz, C<u>H</u>$_{ar}$) and 9.05 (1H, dd, J 8.1 and 0.9Hz, C<u>H</u>$_{ar}$); δ_cCDCl$_3$; 63MHz) 21.9 (<u>C</u>H$_3$), 36.4 (<u>C</u>H), 42.2 (<u>C</u>H$_2$CO), 44.8 (2 x N(<u>C</u>H$_3$)$_2$), 67.6 (<u>C</u>H$_2$O), 106.1 (<u>C</u>H$_{bt}$), 112.8 (<u>C</u>H$_{ar}$), 112.9 (<u>C</u>H$_{ar}$) 118.7 (<u>C</u>H$_{bt}$), 120.2 (<u>C</u>H$_{bt}$), 123.7, 124.6, 125.4 (<u>C</u>H$_{ar}$ x 3), 126.4 (<u>C</u>H$_{ar}$ x 2), 126.4, 126.9 (<u>C</u>H$_{ar}$ x 2), 126.5 (<u>C</u>H$_{ar}$ x 2), 128.3 (<u>C</u>H$_{ar}$ x 2), 128.0, 133.3, 133.3, 142.4, 144.4, 146.5, 153.0, 155.1 (<u>C</u>$_{ar}$ x 8) and 171.2 (C=O); m/z (ESi+, 45kV) 493.2 (100%, [MH]$^+$); m/z (FAB, 3-NOBA) 493 (80%, [MH]$^+$), (FAB, 3-NOBA) Found 493.23656 $C_{29}H_{28}N_6O_2$ requires 493.235197

(*S*)-**143C**

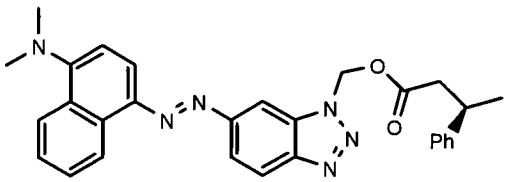
R_f (DCM/Et_2O, 99:1) 0.40; υ_{max} (thin film)/cm^{-1} 3054, 3024 (CH_{ar}), 2964, 2923, 2825 (CH), 1757 (C=O), 1607 (C=C), 1574 (C=C), 1509 (C=C) 1393 and 1136; δ_H ($CDCl_3$; 250MHz) 1.29 (3H, d, J 7Hz, $C\underline{H}_3$), 2.67 (1H, dd, J 15.4 and 7.8Hz, $C\underline{H}_aH_bCO$), 2.74 (1H, dd, J 15.4 and 7.3Hz, $CH_a\underline{H}_bCO$), 3.23–3.37 (1H, m, $C\underline{H}$), 6.51 (1H, d, J 10.0Hz, $C\underline{H}_aH_bO$), 6.58 (1H, d, J 10.0Hz, $CH_a\underline{H}_bO$), 7.07-7.23 (6H, m, Ar$\underline{H}$ (x5) + $C\underline{H}_{ar}$ naphthalene overlapping), 7.57 (1H, ddd, J 8.3, 6.9 and 1.5Hz, $C\underline{H}_{ar}$), 7.65 (1H, ddd, J 8.3, 6.9 and 1.5Hz, $C\underline{H}_{ar}$), 7.95 (1H, d, J 8.3Hz, $C\underline{H}_{ar}$) overlapping with 7.98 (1H, $C\underline{H}_{bt}$), 8.23 (1H, dd, J 8.9 and 1.4Hz, $C\underline{H}_{ar}$), 8.25 (1H, dd, J 9.3 and 1.7Hz, $C\underline{H}_{bt}$) 8.49 (1H, d, J 1Hz, $C\underline{H}_{bt}$) and 9.02 (1H, dd, J 8.4 and 1.5, $C\underline{H}_{ar}$); δ_c($CDCl_3$; 63MHz) 22.2 ($\underline{C}H_3$), 36.7 ($\underline{C}H$), 42.7 ($\underline{C}H_2CO$), 45.3 (2 x $N(\underline{C}H_3)_2$), 75.4 ($\underline{C}H_2O$), 113.1 ($\underline{C}H_{ar}$), 113.6 ($\underline{C}H_{ar}$), 117.8 ($\underline{C}H_{bt}$), 119.6 ($\underline{C}H_{bt}$), 121.2 ($\underline{C}H_{bt}$), 124.2, 125.1, 125.9, 127.0 ($\underline{C}H_{ar}$ x 4), 127.0 ($\underline{C}H_{ar}$ x 2), 127.3 ($\underline{C}H_{ar}$), 128.6 ($\underline{C}_{ar}$), 128.9 ($\underline{C}H_{ar}$ x 2), 133.7, 143.0, 145.3, 145.9, 146.4, 153.1, 155.3 ($\underline{C}_{ar}$ x 7) and 171.0 (C=O); m/z (ESi+, 45kV) 493.2 (100%, $[MH]^+$); m/z (FAB, 3-NOBA) 493 (90%, $[MH]^+$), (FAB, 3-NOBA) Found 493.23550 $C_{29}H_{28}N_6O_2$ requires 493.23519.

A, **B** and **C** were all cleaved and the resulting (*S*)–3 phenylbutyric acid determined according to protocol **5.4.18** to be >99% e.e. – which is in excellent agreement with starting acid.

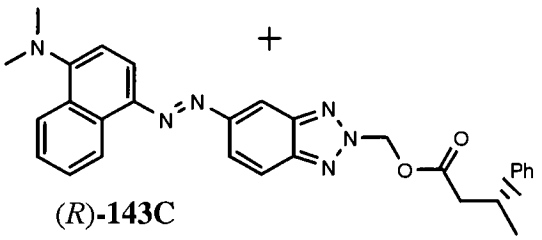
5.4.24. (*R*)-3-Phenyl-butyric acid 5-(4-dimethylamino-napthalen-1-ylazo)-benzotriazol-1-ylmethyl ester (143A), (*R*)-3-Phenyl-butyric acid 6-(4-dimethylamino-napthalen-1-ylazo)-benzotriazol-1-yl methyl ester (143B) and (*R*)-3-Phenyl-butyric acid 5-(4-dimethylamino-napthalen-1-ylazo)-benzotriazol-2-yl methyl ester (143C).



(*R*)-**143A**



(*R*)-**143B**



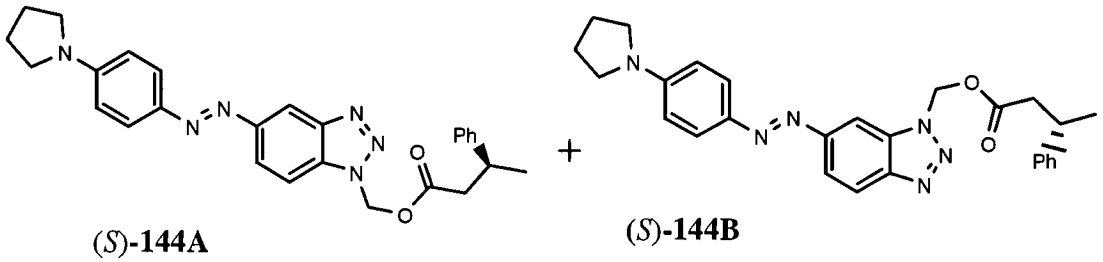
(*R*)-**143C**

The general protocol **5.4.18** was followed with (*R*)-**139A+B** (191.3mg, 0.414mmols) dissolved in 25ml of CH_3CN: DCM (9:1). Crushed K_2CO_3 (8.6mg, 0.062mols, 15mol %) and formaldehyde (37µl, 0.435mmols, 1.05 equiv.) were added and the reaction stirred vigorously overnight at room temperature. Column chromatography eluting with DCM, furnished the desired products as dark red oils; **A+B** (136mg, 66.7%) as a mixture of both regioisomers in the ratio 48:52 (as determined by the integral of peaks at 2.96, 6.45 and 9.0p.p.m) and **C** (13.7 mg, 6.7%). **A+B** (40mg) were taken and separated on a Biotage Flex system eluting at 50:50 (CH_3CN: H_2O) to provide **B** as a deep red oil (5.2mg) and **A** as an orange/red oil (8.7mg).

Analytical data for (*R*)-**143A**, (*R*)-**143B** and (*R*)-**143C** were all comparable to the appropriate (*S*)-enantiomers in **5.4.23**

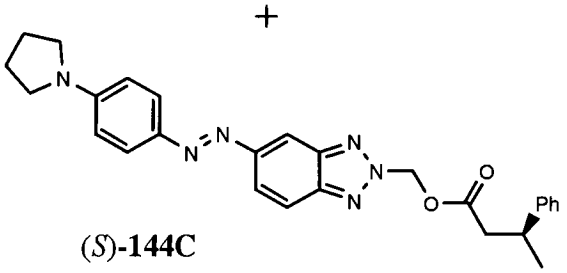
A, **B** and **C** were all cleaved and the resulting (*R*)–3 phenylbutyric acid determined according to protocol **5.4.18** to be >99% e.e.– which is in excellent agreement with starting acid.

5.4.25 (*S*)-3-Phenyl-butyric acid 5-(4-pyrolidin-1-yl-phenylazo)-benzotriazol-1-yl methyl ester (144A), (*S*)-3-phenyl-butyric acid 6-(4-pyrolidin-1-yl-phenylazo)-benzotriazol-1-yl methyl ester (144B) and (*S*)-3-phenyl-butyric acid 5-(4-pyrolidin-1-yl-phenylazo)-benzotriazol-2-yl methyl ester (144C).



(*S*)-**144A** + (*S*)-**144B**

+



(*S*)-**144C**

The general protocol **5.4.18** was followed with (*S*)-**140(A+B)** (195.6mg, 0.45mmols) dissolved in 60ml of CH_3CN: DCM (9:1). Crushed K_2CO_3 (9.6mg, 0.108mols, 24 mol%) and formaldehyde (40μl, 0.473mmols, 1.05 equiv.) were added and the reaction stirred vigorously overnight at room temperature. Column chromatography eluting with DCM, furnished the desired products as dark red oils; **A+B** (138mg, 65%) as a mixture of the regioisomers in the ratio 50:50 (as determined by integral of methyl peaks at 1.31 and 1.32 p.p.m) and **C** (21.4 mg, 10%). **A+B** could not be separated by prep scale HPLC despite extensive method development.

(*S*)-**144A+B**

R_f (DCM/Et_2O, 99:1) 0.25; υ_{max}(thin film)/cm^{-1} 3055 (CH_{ar}), 2966, 2874 (CH), 1751 (C=O), 1603 (C=C), 1517 (C=C), 1384, 1144; δ_H ($CDCl_3$; 250MHz) 1.31 (1.5H, d, J 7.0Hz, $C\underline{H}_3$, one regioisomer), 1.32 (1.5H, d, J 7.0Hz, $C\underline{H}_3$, one regioisomer), 2.12-2.14 (4H, m, $NCH_2C\underline{H}_2$, both regioisomers), 2.67 (1H, dd, J 15.4 and 7.9Hz, $C\underline{H}_aH_bCO$, both

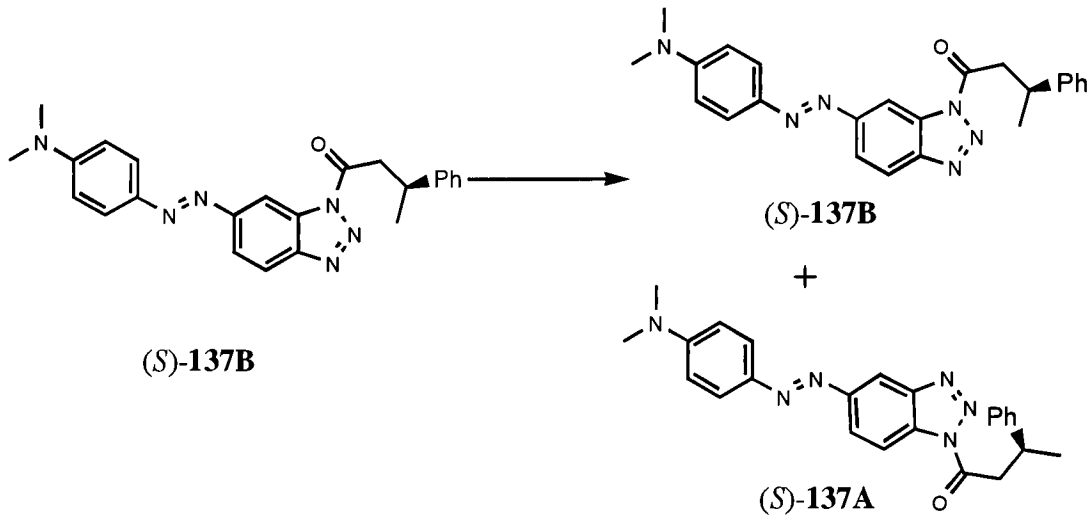
regioisomers), 2.73 (1H, dd, J 15.2 and 7.3Hz, $C\underline{H}_aH_bCO$, both regioisomers), 3.25-3.38 (1H, m, C<u>H</u>, both regioisomers), 3.46-3.49 (4H, m, $NCH_2C\underline{H}_2$, both regioisomers), 6.54 (0.5H, d, J 11Hz, $C\underline{H}_aH_bO$, one regioisomer), 6.57 (0.5H, d, J 11.1Hz, $C\underline{H}_aH_bO$, one regioisomer), 6.61 (0.5H, d, J 11Hz, $CH_a\underline{H}_bO$, one regioisomer), 6.65 (0.5H, d, J 11.1Hz, $CH_a\underline{H}_bO$, one regioisomer), 6.71 (2H, d, J 9Hz, $C\underline{H}_{ar}$ x 2, both regioisomers), 7.09-7.19 (5H, m, Ar<u>H</u>, both regioisomers), 7.67 (0.5H, d, J 9Hz, $C\underline{H}_{bt}$, **A**), 8.00 (1H, d, J 9Hz, 2 x $C\underline{H}_{ar}$, one regioisomer), 8.01 (1H, d, J 9Hz, 2 x $C\underline{H}_{ar}$, one regioisomer), 8.03-8.09 (1.5H, m, overlapping signals from 3 x $C\underline{H}_{bt}$), 8.16 (0.5H, d, J 9.4, $C\underline{H}_{bt}$, **B**), 8.18 (0.5H, dd, J 9 and 1.6Hz, $C\underline{H}_{bt}$, **A**) and 8.55 (0.5H, dd, J 1.6 and 0.8Hz, $C\underline{H}_{bt}$, **A**); δ_c ($CDCl_3$; 63MHz) 22.3, 23.4 ($\underline{C}H_3$, both regioisomers), 25.9 ($NCH_2\underline{C}H_2$, both regioisomers), 36.9 (<u>C</u>H, both regioisomers), 42.8 ($\underline{C}H_2CO$), 48.2 ($N\underline{C}H_2CH_2$, both regioisomers), 68.0, 68.3 ($\underline{C}H_2O$, both regioisomers), 104.5 ($\underline{C}H_{bt}$, **B**), 110.5 ($\underline{C}H_{bt}$, **A**), 111.9, 112.0 ($\underline{C}H_{ar}$ x 2, both regioisomers) 115.5 ($\underline{C}H_{bt}$, **A**), 119.6 ($\underline{C}H_{bt}$, **B**), 120.5 ($\underline{C}H_{bt}$, **B**), 125.9 ($\underline{C}H_{bt}$, **A**), 126.3 ($\underline{C}H_{ar}$, both regioisomers), 126.9 ($\underline{C}H_{ar}$ x 2, both regioisomers), 127.0 ($\underline{C}H_{ar}$ x 2, both regioisomers), 128.9 ($\underline{C}H_{ar}$ x 2, both regioisomers), 133.2, 133.9, 143.5, 143.7, 144.9, 145.0, 146.7, 147.0, 150.7, 150.9, 151.2 and 153.7 ($\underline{C}_{ar}$ x 12, both regioisomers), 171.7 and 171.8 (C=O, both regioisomers); m/z (ESi+, 45kV) 469.4 (100%, $[MH]^+$), 293 (10%, $[MH–CH_2OCOCH_2CHPhCH_3]^+$); m/z (FAB, NOBA) 469 (10%, $[MH]^+$), 293 (30%, $[MH–CH_2OCOCH_2CHPhCH_3]^+$); (FAB, 3-NOBA) Found 469.23523 $C_{27}H_{28}N_6O_2$ requires 469.23520.

(*S*)-**144C**

R_f (DCM/Et_2O, 99:1) 0.44; υ_{max}(thin film)/cm^{-1} 3029 (CH_{ar}), 2924, 2825 (CH), 1755 (C=O), 1603 (C=C), 1384 and 1135; δ_H ($CDCl_3$; 250MHz) 1.27 (3H, d, J 7Hz, $C\underline{H}_3$), 2.03-2.09 (4H, m, $NCH_2C\underline{H}_2$), 2.67 (1H, dd, J 15.4 and 7.9Hz, $C\underline{H}_aH_bCO$), 2.73 (1H, dd, J 15.4 and 7.3Hz, $CH_a\underline{H}_bCO$), 3.27–3.32 (1H, m, C<u>H</u>), 3.39-3.45 (4H, m, $NC\underline{H}_2CH_2$), 6.48 (1H, d, J 10.0Hz, $C\underline{H}_aH_bO$), 6.55 (1H, d, J 10.0Hz, $CH_a\underline{H}_bO$), 6.63 (2H, d, J 9.2Hz, $C\underline{H}_{ar}$ x 2), 7.09-7.22 (5H, m, Ar<u>H</u>), 7.86 (1H, d, J 0.7Hz, $C\underline{H}_{bt}$, obscured by signal at 7.92 p.p.m), 7.92 (2H, d, J 9.1Hz, $C\underline{H}_{ar}$ x 2), 8.09 (1H, dd, J 9.3 and 1.7Hz, $C\underline{H}_{bt}$) and 8.28 (1H, dd, J 1.6 and 0.8Hz, $C\underline{H}_{bt}$); δ_c ($CDCl_3$; 63MHz) 21.7 ($\underline{C}H_3$), 25.4 (2 x

$NCH_2\underline{C}H_2$), 36.7 (<u>C</u>H), 42.2 ($\underline{C}H_2CO$), 47.7 (2 x $N\underline{C}H_2CH_2$), 74.9 ($\underline{C}H_2O$), 111.4 ($\underline{C}H_{ar}$ x 2), 115.1 ($\underline{C}H_{bt}$), 118.7 ($\underline{C}H_{bt}$), 120.9 ($\underline{C}H_{bt}$), 125.5 ($\underline{C}H_{ar}$ x 2), 126.4 ($\underline{C}H_{ar}$), 126.5 ($\underline{C}H_{ar}$ x 2), 128.4 ($\underline{C}H_{ar}$ x 2), 143.1, 144.8, 145.5, 150.2, 152.4, 153.1 ($\underline{C}_{ar}$ x 6) and 170.5 (C=O); m/z (ESi+, 45kV) 491.3 (100%, [MNa]), 469.4 (50%, $[MH]^+$), 293 (10%, $[MH - CH_2OCOCH_2CHPhCH_3]^+$); m/z (FAB, 3-NOBA) 469 (10%, $[MH]^+$), (FAB, 3-NOBA) Found 469.23512 $C_{27}H_{28}N_6O_2$ requires 469.235199.

5.4.26 Treatment of (*S*)-137B with base



(*S*)-**137B** (5mg, $1.2x10^{-5}$mols) was dissolved in CH_3CN: DCM (9:1, 2ml). To this was added potassium carbonate (0.1mg) and the mixture stirred for sixteen hours at room temperature. At various time points the solvent was removed under reduced pressure and the crude mixture analysed by NMR in $CDCl_3$. After NMR analysis the deuterated solvent was removed under reduced pressure and the crude mixture resuspended in CH_3CN: DCM (9:1, 2ml). After sixteen hours the solvent was removed under reduced pressure and the crude mixture purified by column chromatography eluting with DCM to provide the title product as a mixture of regioisomers (4.0mg, 80%).

δ_H ($CDCl_3$; 200 MHz) (only benzotriazole signals reported.)

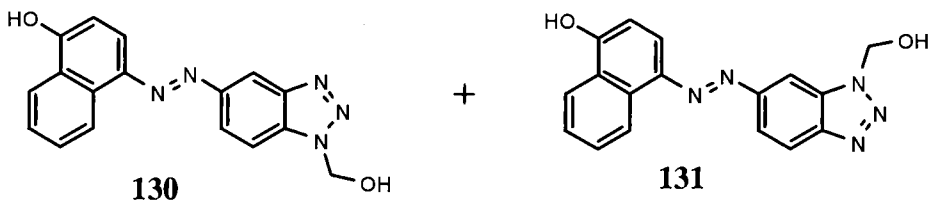
Initial - δ_H ($CDCl_3$; 200 MHz) 7.94 (1H, dd, J 9.0 and 0.8Hz, C<u>H</u>$_{bt}$), 8.07 (1H, d, J 9.0Hz, C<u>H</u>$_{bt}$) and 8.56 (1H, d, J 0.8Hz, C<u>H</u>$_{bt}$).

After 1 hour – δ_H ($CDCl_3$; 200 MHz) 7.94 (1H, dd, J 9.0 and 0.8Hz, C<u>H</u>$_{bt}$), 8.07 (1H, d, J 9.0Hz, C<u>H</u>$_{bt}$) and 8.56 (1H, d, J 0.8Hz, C<u>H</u>$_{bt}$).

After 16 hours - δ_H ($CDCl_3$; 200 MHz) 7.97 (0.57H, d, J 8.2Hz, C<u>H</u>$_{bt}$), 8.06 (0.57H, d, J 8.2Hz, C<u>H</u>$_{bt}$), 8.17 (0.43H, dd, J 8.2Hz, C<u>H</u>$_{bt}$), 8.21 (0.43H, d, J 8.2Hz, C<u>H</u>$_{bt}$), 8.42 (0.43H, s, C<u>H</u>$_{bt}$) and 8.59 (0.57H, s, C<u>H</u>$_{bt}$).

The ratio of regioisomers, after 16 hours incubation with K_2CO_3, was calculated as 43:57 (**A**: **B**) by integration of peaks at 8.42 and 8.59 p.p.m respectively.

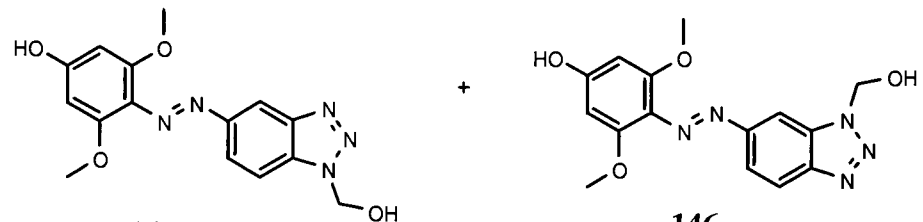
5.4.27 Attempted synthesis of 4-(1-Hydroxymethyl-1*H*-benzotriazol-5-ylazo-)-naphthalen-1-ol (130) and 4-(1-Hydroxymethyl-1*H*-6-ylazo)-napthalen-1-ol (131)



130 + **131**

120 (250mg, 0.87mmols) was suspended in glacial acetic acid (2ml) with H_2O (2ml) and acetone (2ml). The reaction was heated to 70°C and formaldehyde (64.9µl, 1.74mmols, 2 equiv.) was added. The reaction was heated for a further 30mins then allowed to cool. On cooling a dark brown precipitate was formed which was collected and dried under high vacuum to give a dark brown powder (0.201g, 72%). The crude product was analysed by reverse phase HPLC eluting with 85% MeOH/15% H_2O (no TFA added). Under these conditions the R_t of the parent dye was 3.0mins. The product was used without further purification

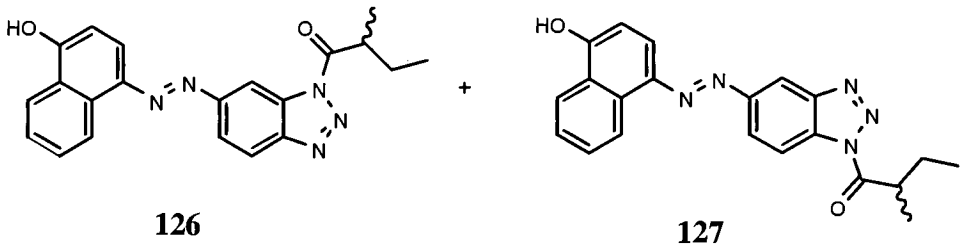
5.4.28 Attempted synthesis of 4-(1-Hydroxymethyl-1*H*-benzotriazol-5-ylazo)-3,5-dimethoxy-phenol (145) and 4-(1-Hydroxymethyl-1*H*-benzotriazol-6-ylazo)-3,5-dimethoxy-phenol (146).



145 + **146**

119 (0.165g, 0.55mmols) was dissolved in the minimum amount of acetic acid: H_2O (2:1, approx 20ml). To this was added formaldehyde (0.041ml, 0.55mmols, 1 equiv) and the reaction mixture was allowed to stir for three hours at room temperature. The red precipitate formed was filtered off, washed with cold ether (10ml) and dried in a vacuum dessicator over P_2O_5 under reduced pressure. Further purification was unsuccessful.

5.4.29 Attempted synthesis of 1-[6-(4-Hydroxy-naphthalen-1-ylazo)-benzotriazol-1-yl]-2-methyl-butan-1-one (126) and 1-[5-(4-hydroxy-naphthalen-1-ylazo)-benzotriazol-1-yl]-2-methyl-butan-1-one (127).



126 **127**

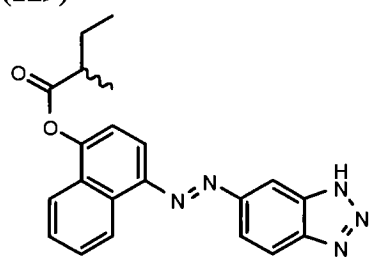
120 (200mg, 0.692mmols) was dissolved in 'anhydrous' DCM under an inert atmosphere of nitrogen and cooled to 0°C. To this was added triethylamine (0.115ml, 1.2equiv) followed by (*R/S*)-2-methyl butyryl chloride **109** (0.129ml, 1.5equiv.) and the reaction mixture was stirred for 1hour. HPLC analysis indicated that the azo dye (eluting at 6mins) had been converted to less polar components at 25, 28, 42 and 47minutes. The reaction was quenched by addition of HCl (2M, 10ml) and further DCM (50ml) was added. The organic layer was separated, washed with H_2O (20ml), dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to give a deep red oil. The crude mixture was dissolved in CH_3CN (5ml) and purified by Prep scale HPLC collecting 4 fractions at 25, 28, 42 and 47 minutes respectively. The organic solvent was removed from each fraction and the product extracted with DCM, dried over $MgSO_4$, filtered and the solvent removed in vacuo to give the four fractions as red oils; 25mins (5mg, 1.9%), 28mins (0.7mg, 0.3%), 42mins (1.8mg, 0.7%) and 47mins (53mg, 20.5%).

HPLC method:

Flowrate:	$10ml/min^{-1}$
Detection :	254 and 410nm
Injection volume:	1ml
Temperature:	Ambient

Time (mins)	% H_2O	% CH_3CN
0	50	50
20	20	80
40	20	80
60	5	95

5.4.30 Isolation of (*R/S*)-2-methylbutyric acid 4-(1*H*-benzotriazol-6-ylazo)-napthalen-1-yl ester (129)



129

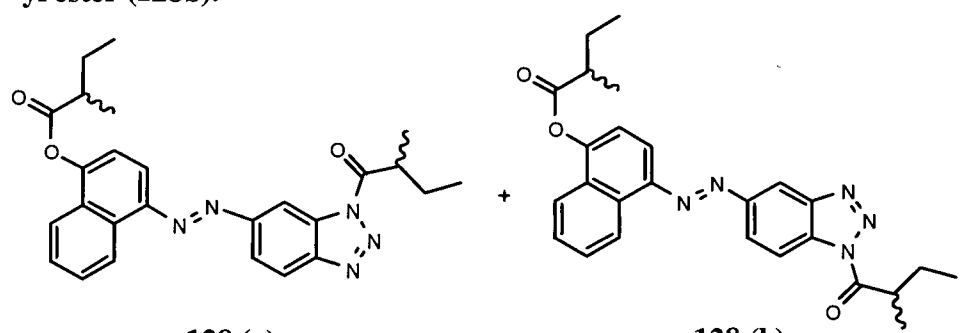
R_t 25 mins; υ_{max}(thin film)/cm^{-1} 3054 (CH_{ar}), 2986, 2936, 2878 (CH), 1752 (C=O), 1635 (C=C), 1574 (C=C), 1507, 1265; δ_H ($CDCl_3$; 250MHz) 1.15 (3H, t, J 7.4Hz, $CH_2C\underline{H}_3$), 1.48 (3H, d, J 6.9Hz, $CHC\underline{H}_3$), 1.72-1.86 (1H, m, $C\underline{H}_aH_bCH$), 1.97-2.11 (1H, m, $CH_a\underline{H}_bCH$), 2.89 (1H, tq, J 6.9 and 6.9Hz, $C\underline{H}CH_3$) 7.31 (1H, d, J 8.4Hz, $C\underline{H}_{ar}$), 7.56 – 7.63 (2H, m, $C\underline{H}_{ar}$ x 2), 7.73 (1H, d, J 8.9, $C\underline{H}_{ar}$), 7.82 (1H, d, J 8.4Hz, $C\underline{H}_{ar}$), 7.92 (1H, br d, J 9.2Hz, $C\underline{H}_{bt}$), 8.09 (1H, br d, J 9.2Hz, $C\underline{H}_{bt}$), 8.46 (1H, br s, $C\underline{H}_{bt}$) and 8.87 (1H, br d, 8.8Hz, $C\underline{H}_{ar}$); δ_C ($CDCl_3$; 63MHz) 12.2 ($CH_2\underline{C}H_3$), 17.2 ($\underline{C}H_3$), 27.3 ($\underline{C}H_2CH_3$), 42.0 ($\underline{C}H$) 112.3 ($\underline{C}H_{bt}$), 113.8 ($\underline{C}H_{ar}$), 115.3 and 118.5 ($\underline{C}H_{bt}$), 120.3 ($\underline{C}H_{ar}$), 121.6 ($\underline{C}H_{ar}$), 124.1 ($\underline{C}H_{ar}$), 127.6 ($\underline{C}H_{ar}$), 127.8 ($\underline{C}_{ar}$), 127.9 ($\underline{C}H_{ar}$), 129.5, 133.0, 143.0, 145.5, 149.8, 151.5 ($\underline{C}_{ar}$ x 6); m/z (ESi -ve, 45Kv) 372.0 (100%, $[M-H]^-$); m/z (FAB+ve, 3-NOBA) 374 (70%, $[MH]^+$), 290 (60%, $[M-(CH_3CH_2CHCH_3C=O)]^+$,

120 (40%, $[BtH]^+$) and 77 (60%, $[Ph]^+$); m/z (FAB+ve, 3-NOBA) Found 374.16284 $C_{21}H_{20}N_5O_2$ requires 374.16398

Fraction at 28 mins – Weak NMR due to lack of material; m/z (ESi -ve, 45Kv) 372.1 (100%, $[M-H]^-$).

Fraction at 42 mins – impure NMR; m/z (ESi -ve, 45Kv) 372.1 (100%, $[M-H]^-$)

5.4.31 Isolation of (*R/S*)-2-methylbutyric acid 4-[3-(2-methyl-butyryl)-1*H*-benzotriazol-6-ylazo]-naphthalen-1-yl ester (128a) and (*R/S*)-2-methyl-butyric acid 4-[1-(2-methyl-butyryl)-1*H*-benzotriazol-5-ylazo]-naphthalen-1-yl ester (128b).



128 (a) **128 (b)**

R_t 47 mins; υ_{max}(thin film)/cm^{-1} 3054 (CH_{ar}), 2969, 2936, 2877 (CH), 1758 (C=O), 1738(C=O), 1589(C=C), 1574 (C=C), 1508, 1461; δ_H ($CDCl_3$; 250MHz) 1.01, 1.04 and 1.17 (6H, t, J 7.4Hz, $CH_2C\underline{H}_3$ x 3 overlapping), 1.44 (4.5H, d, J 6.9Hz, $CHC\underline{H}_3$ x 2 overlapping) 1.45 (1.5H, d, J 6.9Hz, $CHC\underline{H}_3$) 1.64-1.88 (2H, m, $C\underline{H}_aH_bCH$ x 2 overlapping), 1.92-2.12 (2H, m, $CH_a\underline{H}_bCH$ x 2 overlapping), 2.84 (1H, tq, J 6.9 and 6.9Hz, $OC=OC\underline{H}CH_3$), 4.05 and 4.07 (1H, tq, J 6.9 and 6.9 Hz, $NC=OC\underline{H}CH_3$, overlapping) 7.34 (1H, d, J 8.4Hz, $C\underline{H}_{ar}$), 7.56 – 7.70 (2H, ddd, J 8.3, 6.8 and 1.0Hz, $C\underline{H}_{ar}$ x 2 overlapping), 7.91-7.97 (2H, m, $C\underline{H}_{ar}$ x 2), 8.19 (1H, 2 x d overlapping, J 8.9Hz, $C\underline{H}_{bt}$), 8.38 (1H, m, $C\underline{H}_{bt}$ x 2), 8.70 (0.5H, br s, $C\underline{H}_{bt}$, one regioisomer), 8.86 (0.5H, br s, CH_{bt}, one regioisomer) and 8.98 (1H, 2 x d overlapping, $C\underline{H}_{ar}$); δ_C ($CDCl_3$; 63MHz) 12.0, 12.2 ($CH_2\underline{C}H_3$), 17.1, 17.2, 17.2 ($\underline{C}H_3$), 27.2, 27.3 ($\underline{C}H_2CH_3$), 41.0, 42.0 ($\underline{C}H$), 110.9, 112.6, 112.7, 115.3, 116.6, 118.5, 118.6, 120.9, 121.3, 121.7, 121.7, 124.1, 124.1, 125.0, 127.5, 127.5 ($\underline{C}H_{ar}$ + $\underline{C}H_{bt}$ x 16), 127.9, 127.9 (2 x $\underline{C}_{ar}$) 128.0, 128.1

($\underline{C}H_{ar}$), 132.4, 132.9, 133.1, 133.2, 145.4, 145.5, 147.3, 150.2, 150.5, 151.7, 152.0, 154.3 ($\underline{C}_{ar}$ x 12), 175.1, 175.2 (C=O, *N*-acyl) and 176.6 (C=O, ester); m/z (FAB +ve, 3-NOBA) 458.0 (55%, $[M-H]^+$); 374 (10%, $[MH-(CH_3CH_2CHCH_3C=O)]^+$, 290.0 (30%, $[M-(CH_3CH_2CHCH_3C=O)x\ 2]^+$), 120 (25%, $[BtH]^+$) and 77 (60%,$[Ph]^+$); m/z (FAB, NOBA) Found 458.21927 $C_{26}H_{28}N_5O_3$ requires 458.21933.

5.5 Measurment of SERRS spectra of dyes (121-123)

SERRS spectra were measured at an excitation wavelength of 514nm. Silver colloid (0.5ml) was added to the cuvette followed by monoazo-benzotriazole dye (*e.g.* **121**, 0.1ml, 1 x 10^{-5}M in pH 8 HEPES buffer). The resulting solution was stirred and analysed immediately. Spectra were acquired for 1s accumulation time every 5 seconds.

Measurement of SERRS spectra of blocked ester dye (*S*)-141A

SERRS spectra were measured at an excitation wavelength of 514nm. Silver colloid (0.5ml) was added to the cuvette followed by the appropriate enzyme solution (e.g. PCL, 0.5ml, 1mg/ml in pH 8 HEPES buffer). To this suspension was added (*S*)-**141A** (0.1ml, 1 x 10^{-5}M in pH 8 HEPES buffer) and the resulting solution analysed immediately. Spectra were acquired for 1s accumulation time every 5 seconds for the duration of the hydrolysis reaction.

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7.Appendices

7.1. X-ray crystal structure for [4-(1H-benzotriazol-6-ylazo)-3-methoxy-phenyl]-dimethyl-amine (122).

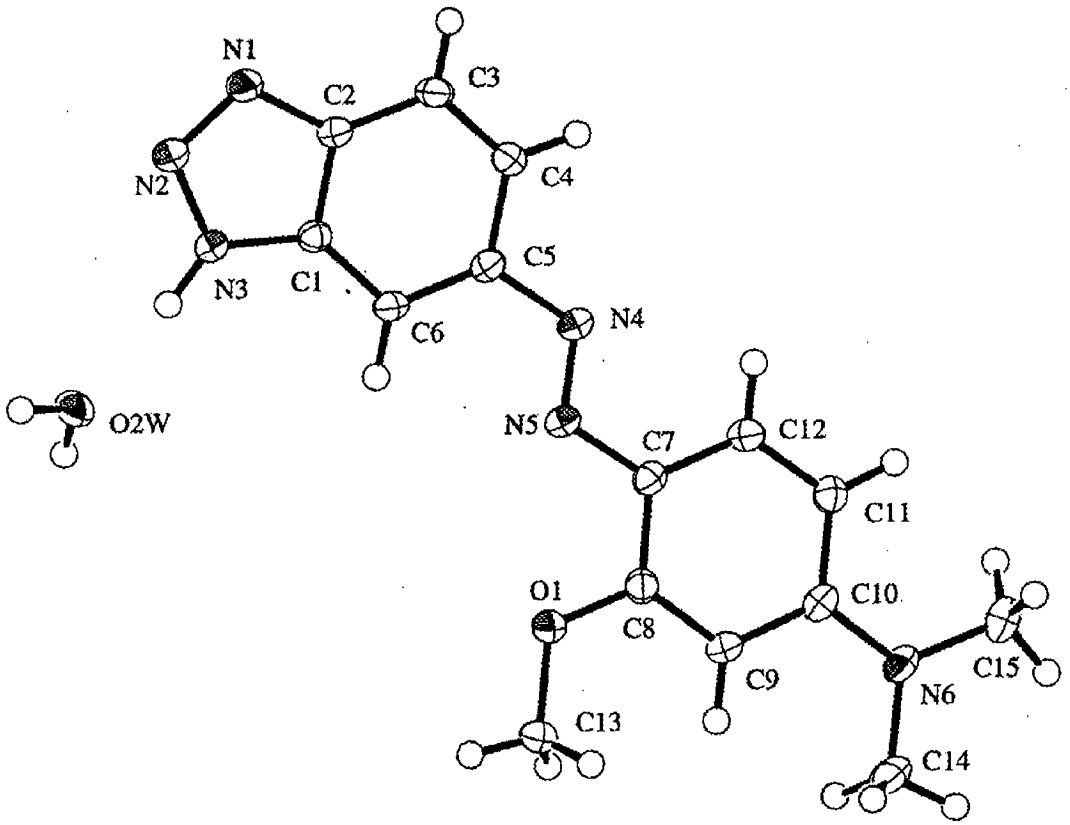


Table1 Crystal data and structure refinement for/disk2/texsan/extras/ciftab.

Identification code	dg10
Empirical formula	C_{15} H_{18} N_6 O_2
Formula weight	314.35
Temperature	123(2) K
Wavelength	0.71073 A
Crystal system, space group	triclinic PT
Unit cell dimensions	a = 7.1142(2) A alpha = 90.4490(10) deg. b = 10.3909(3) A beta = 104.6160(10) deg. c = 10.7890(3) A gamma = 99.1340(10) deg.
Volume	761.00(4) A^3
Z, Calculated density	2, 1.372 Mg/mA3
Absorption coefficient	0.096 mm^{-1}
Crystal size	0.20 x 0.10 x 0.10 mm
Theta range for data collection	3.00 to 27.03 deg.
Index ranges	-9<=h<=8, -12<=k<=13,13<=1<=13
Reflections collected / unique	5941 / 3317 [R(int) = 0.0169]
Completeness to 2theta = 27.03	99.5%
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	3317 / 0 / 223
Goodness-of-fit on F^2	1.035
Final R indices [I>2sigma(I)]	R1 = 0.0408, wR2 = 0.1007
R indices (all data)	R1 = 0.0549, wR2 = 0.1088
Largest diff. peak and hole	0.233 and -0.199 e.A^-3

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (A^2 x 10^3) for /disk2/texsan/extras/ciftab. U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

	x	y	z	U(eq)
O(1)	1762(1)	-2515(1)	-6333(1)	27(1)
O(2W)	-76(2)	1795(1)	-11100(1)	29(1)
N(1)	-5926(2)	2241(1)	-9905(1)	22(1)
N(2)	-4569(2)	2367(1)	-10544(1)	24(1)
N(3)	-3116(2)	1723(1)	-9952(1)	22(1)
N(4)	-2695(2)	-826(1)	-6024(1)	23(1)
N(5)	-1292(2)	-1368(1)	-6199(1)	22(1)
N(6)	1935(2)	-4510(1)	-2289(1)	30(1)
C(1)	-3525(2)	1154(1)	-8897(1)	19(1)
C(2)	-5344(2)	1491(1)	-8877(1)	19(1)
C(3)	-6278(2)	1056(1)	-7917(1)	23(1)
C(4)	-5308(2)	317(1)	-6997(1)	23(1)
C(5)	-3461(2)	-25(1)	-7034(1)	21(1)
C(6)	-2531(2)	389(1)	-7977(1)	20(1)
C(7)	-566(2)	-2169(1)	-5205(1)	21(1)
C(8)	1057(2)	-2759(1)	-5276(1)	21(1)
C(9)	1879(2)	-3530(1)	-4315(1)	23(1)
C(10)	1086(2)	-3776(1)	-3251(1)	23(1)
C(11)	-603(2)	-3231(1)	-3210(1)	23(1)
C(12)	-1374(2)	-2447(1)	-4161(1)	22(1)
C(13)	3357(2)	-3143(1)	-6466(1)	27(1)
C(14)	3615(2)	-5103(2)	-2383(2)	34(1)
C(15)	1067(2)	-4842(1)	-1231(1)	30(1)

Table 3. Bond lengths [A] and angles [deg] for /disk2/texsan/extras/ciftab

O(1)-C(8)	1.3674(15)
O(1)-C(13)	1.4316(15)
O(2W)-H(1W)	0.88(2)
O(2W)-H(2W)	0.87(2)
N(1)-N(2)	1.3117(15)
N(1)-C(2)	1.3760(16)
N(2)-N(3)	1.3480(15)
N(3)-C(1)	1.3611(17)
N(3)-H(3)	0.940(18)
N(4)-N(5)	1.2731(15)
N(4)-C(5)	1.4263(16)
N(5)-C(7)	1.4056(16)
N(6)-C(10)	1.3664(16)
N(6)-C(15)	1.4481(18)
N(6)-C(14)	1.4540(18)
C(1)-C(6)	1.3979(17)
C(1)-C(2)	1.3984(17)
C(2)-C(3)	1.4062(18)
C(3)-C(4)	1.3734(18)
C(3)-H(3)	0.9500
C(4)-C(5)	1.4249(18)
C(4)-H(4)	0.9500
C(5)-C(6)	1.3831(18)
C(6)-H(6)	0.9500
C(7)-C(12)	1.4003(18)
C(7)-C(8)	1.4079(17)
C(8)-C(9)	1.3836(17)
C(9)-C(10)	1.4101(19)
C(9)-H(9)	0.9500
C(10)-C(11)	1.4172(18)
C(11)-C(12)	1.3744(18)
C(11)-H(11)	0.9500
C(12)-H(12)	0.9500
C(13)-H(13A)	0.9800
C(13)-H(13B)	0.9800
C(13)-H(13C)	0.9800
C(14)-H(14A)	0.9800
C(14)-H(14B)	0.9800
C(14)-H(14C)	0.9800
C(15)-H(15A)	0.9800
C(15)-H(15B)	0.9800
C(15)-H(15C)	0.9800

C(8)-O(1)-C(13)	117.85(10)
H(1W)-O(2W)-H(2W)	107.3(19)
N(2)-N(1)-C(2)	108.06(10)
N(1)-N(2)-N(3)	108.86(10)
N(2)-N(3)-C(1)	110.62(11)
N(2)-N(3)-H(3)	118.2(11)
C(1)-N(3)-H(3)	131.2(11)
N(5)-N(4)-C(5)	114.17(11)
N(4)-N(5)-C(7)	113.65(11)
C(10)-N(6)-C(15)	121.40(12)
C(10)-N(6)-C(14)	120.12(12)
C(15)-N(6)-C(14)	118.11(11)
N(3)-C(1)-C(6)	133.34(12)
N(3)-C(1)-C(2)	103.97(11)
C(6)-C(1)-C(2)	122.69(11)
N(1)-C(2)-C(1)	108.49(11)
N(1)-C(2)-C(3)	130.68(11)
C(1)-C(2)-C(3)	120.82(11)
C(4)-C(3)-C(2)	117.13(11)

C(4)-C(3)-H(3)	121.4
C(2)-C(3)-H(3)	121.4
C(3)-C(4)-C(5)	121.43(12)
C(3)-C(4)-H(4)	119.3
C(5)-C(4)-H(4)	119.3
C(6)-C(5)-C(4)	122.00(12)
C(6)-C(5)-N(4)	124.18(11)
C(4)-C(5)-N(4)	113.81(11)
C(5)-C(6)-C(1)	115.90(11)
C(5)-C(6)-H(6)	122.1
C(1)-C(6)-H(6)	122.1
C(12)-C(7)-N(5)	124.56(11)
C(12)-C(7)-C(8)	117.46(11)
N(5)-C(7)-C(8)	117.97(11)
O(1)-C(8)-C(9)	122.72(11)
O(1)-C(8)-C(7)	116.23(11)
C(9)-C(8)-C(7)	121.04(12)
C(8)-C(9)-C(10)	120.99(12)
C(8)-C(9)-H(9)	119.5
C(10)-C(9)-H(9)	119.5
N(6)-C(10)-C(9)	120.73(12)
N(6)-C(10)-C(11)	121.36(12)
C(9)-C(10)-C(11)	117.90(12)
C(12)-C(11)-C(10)	120.15(12)
C(12)-C(11)-H(11)	119.9
C(10)-C(11)-H(11)	119.9
C(11)-C(12)-C(7)	122.36(12)
C(11)-C(12)-H(12)	118.8
C(7)-C(12)-H(12)	118.8
O(1)-C(13)-H(13A)	109.5
O(1)-C(13)-H(13B)	109.5
H(13A)-C(13)-H(13B)	109.5
O(1)-C(13)-H(13C)	109.5
H(13A)-C(13)-H(13C)	109.5
H(13B)-C(13)-H(13C)	109.5
N(6)-C(14)-H(14A)	109.5
N(6)-C(14)-H(14B)	109.5
H(14A)-C(14)-H(14B)	109.5
N(6)-C(14)-H(14C)	109.5
H(14A)-C(14)-H(14C)	109.5
H(14B)-C(14)-H(14C)	109.5
N(6)-C(15)-H(15A)	109.5
N(6)-C(15)-H(15B)	109.5
H(15A)-C(15)-H(15B)	109.5
N(6)-C(15)-H(15C)	109.5
H(15A)-C(15)-H(15C)	109.5
H(15B)-C(15)-H(15C)	109.5

Symmetry transformations used to generate equivalent atoms:

Table 4. Anisotropic displacement parameters (A^2 x 10^3) for /disk2/texsan/extra
The anisotropic displacement factor exponent takes the form:
-2 pi^2 [h^2 a*^2 U11 + ... + 2 h k a* b* U12]

	U11	U22	U33	U23	U13	U12
O(1)	25(1)	35(1)	27(1)	11(1)	11(1)	14(1)
O(2W)	20(1)	43(1)	27(1)	9(1)	8(1)	9(1)
N(1)	21(1)	26(1)	21(1)	3(1)	5(1)	8(1)
N(2)	22(1)	26(1)	24(1)	5(1)	5(1)	7(1)
N(3)	21(1)	23(1)	23(1)	6(1)	6(1)	7(1)
N(4)	22(1)	24(1)	24(1)	4(1)	5(1)	9(1)
N(5)	20(1)	22(1)	22(1)	4(1)	4(1)	7(1)
N(6)	36(1)	33(1)	26(1)	13(1)	10(1)	17(1)
C(1)	19(1)	18(1)	21(1)	0(1)	5(1)	3(1)
C(2)	18(1)	20(1)	20(1)	0(1)	3(1)	6(1)
C(3)	20(1)	27(1)	24(1)	1(1)	6(1)	9(1)
C(4)	23(1)	27(1)	22(1)	3(1)	9(1)	7(1)
C(5)	22(1)	20(1)	20(1)	2(1)	3(1)	6(1)
C(6)	17(1)	20(1)	24(1)	1(1)	4(1)	6(1)
C(7)	20(1)	21(1)	20(1)	3(1)	3(1)	5(1)
C(8)	21(1)	22(1)	21(1)	2(1)	6(1)	3(1)
C(9)	20(1)	24(1)	25(1)	4(1)	5(1)	8(1)
C(10)	26(1)	18(1)	22(1)	3(1)	3(1)	5(1)
C(11)	26(1)	24(1)	21(1)	3(1)	7(1)	5(1)
C(12)	21(1)	23(1)	22(1)	1(1)	4(1)	6(1)
C(13)	22(1)	34(1)	30(1)	7(1)	10(1)	11(1)
C(14)	39(1)	36(1)	32(1)	12(1)	7(1)	20(1)
C(15)	36(1)	28(1)	25(1)	8(1)	6(1)	6(1)

Table 5. Hydrogen coordinates (x 10^4) and isotropic displacement parameters (A^2 x 10^3) for /disk2/texsan/extras/ciftab.

	x	y	z	U(eq)
H(1W)	-260(3)	1870(2)	-11930(2)	62(6)
H(2W)	1190(3)	1990(2)	-10750(2)	59(6)
H(3)	-2060(3)	1704(17)	-10325(17)	42(5)
H(3)	-7528	1264	-7907	27
H(4)	-5880	29	-6321	28
H(6)	-1294	168	-7997	24
H(9)	2994	-3899	-4374	27
H(11)	-1204	-3409	-2523	28
H(12)	-2494	-2081	-4109	27
H(13A)	4523	-2833	-5764	40
H(13B)	3655	-2936	-7288	40
H(13C)	2985	-4090	-6439	40
H(14A)	3253	-5679	-3160	51
H(14B)	4013	-5613	-1629	51
H(14C)	4713	-4418	-2424	51
H(15A)	795	-4049	-861	45
H(15B)	1985	-5244	-574	45
H(15C)	-165	-5458	-1542	45

Table 6. Torsion angles [deg] for /disk2/texsan/extras/ciftab.

C(2)-N(1)-N(2)-N(3)	-0.27(14)
N(1)-N(2)-N(3)-C(1)	0.13(14)
C(5)-N(4)-N(5)-C(7)	-179.23(10)
N(2)-N(3)-C(1)-C(6)	179.61(13)
N(2)-N(3)-C(1)-C(2)	0.07(14)
N(2)-N(1)-C(2)-C(1)	0.32(14)
N(2)-N(1)-C(2)-C(3)	-178.81(13)
N(3)-C(1)-C(2)-N(1)	-0.23(13)
C(6)-C(1)-C(2)-N(1)	-179.84(11)
N(3)-C(1)-C(2)-C(3)	179.00(11)
C(6)-C(1)-C(2)-C(3)	-0.61(19)
N(1)-C(2)-C(3)-C(4)	-179.55(12)
C(1)-C(2)-C(3)-C(4)	1.41(19)
C(2)-C(3)-C(4)-C(5)	-1.83(19)
C(3)-C(4)-C(5)-C(6)	1.5(2)
C(3)-C(4)-C(5)-N(4)	-178.25(12)
N(5)-N(4)-C(5)-C(6)	-14.22(18)
N(5)-N(4)-C(5)-C(4)	165.51(11)
C(4)-C(5)-C(6)-C(1)	-0.58(18)
N(4)-C(5)-C(6)-C(1)	179.12(11)
N(3)-C(1)-C(6)-C(5)	-179.30(13)
C(2)-C(1)-C(6)-C(5)	0.17(18)
N(4)-N(5)-C(7)-C(12)	4.81(18)
N(4)-N(5)-C(7)-C(8)	-176.77(11)
C(13)-O(1)-C(8)-C(9)	3.11(18)
C(13)-O(1)-C(8)-C(7)	-177.37(11)
C(12)-C(7)-C(8)-O(1)	177.28(11)
N(5)-C(7)-C(8)-O(1)	-1.25(17)
C(12)-C(7)-C(8)-C(9)	-3.18(19)
N(5)-C(7)-C(8)-C(9)	178.28(11)
O(1)-C(8)-C(9)-C(10)	-178.88(12)
C(7)-C(8)-C(9)-C(10)	1.6(2)
C(15)-N(6)-C(10)-C(9)	-176.00(13)
C(14)-N(6)-C(10)-C(9)	-3.1(2)
C(15)-N(6)-C(10)-C(11)	4.5(2)
C(14)-N(6)-C(10)-C(11)	177.37(13)
C(8)-C(9)-C(10)-N(6)	-178.27(12)
C(8)-C(9)-C(10)-C(11)	1.28(19)
N(6)-C(10)-C(11)-C(12)	177.00(13)
C(9)-C(10)-C(11)-C(12)	-2.55(19)
C(10)-C(11)-C(12)-C(7)	1.0(2)
N(5)-C(7)-C(12)-C(11)	-179.66(12)
C(8)-C(7)-C(12)-C(11)	1.91(19)

Symmetry transformations used to generate equivalent atoms:

Hydrogen bonds with H..A < r(A) + 2.000 Angstroms and <DHA > 110 deg.

D-H	d(D-H)	d(H..A)	<DHA	d(D..A)	A
O2W-H1W	0.876	2.085	151.00	2.882	O1 [-x, -y, -z-2]
O2W-H1W	0.876	2.620	140.77	3.344	N5 [-x, -y, -z-2]
O2W-H2W	0.872	1.999	174.11	2.868	N1 [x+1, y, z]
N3-H3	0.940	1.805	175.13	2.742	O2W

7.2 X-ray crystal structural data for (*S*)-1-[6-(4-Dimethylamino-phenylazo)-benzotriazol-1-yl]-3-phenyl-butan-1-one (137B)

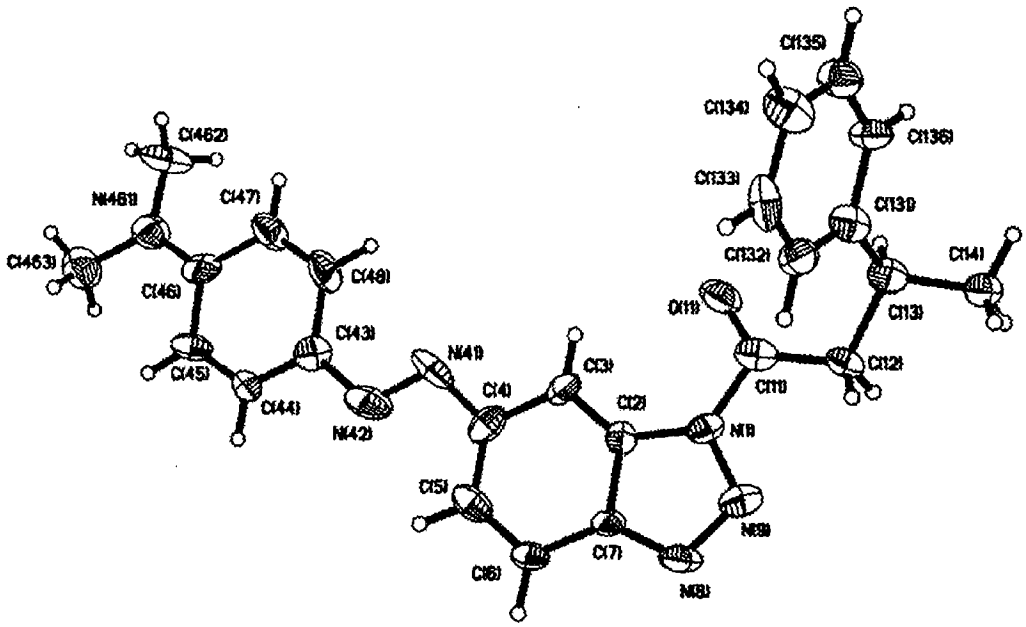


Table 1. Crystal data and structure refinement for alw307.

Contact Andy Parkin, a.parkin@ed.ac.uk

A. CRYSTAL DATA

Empirical formula	C_{24} H_{24} N_6 0
N3C6H3 1-(CO CH2 CHPh CH3) 4-(N2 C6H4 NMe2)	
Formula weight	412.49
Wavelength	0.71073 A
Temperature	150(2) K
Crystal system	Monoclinic
Space group	P2(1)
Unit cell dimensions	a = 9.152(8) A alpha = 90 deg. b = 6.931(6) A beta =104.286(14)deg. c = 16.919(15) A gamma = 90 deg.
Volume	1040.0(16) A^3
Number of reflections for cell	361 (2 < theta < 16 deg.)
Z	2
Density (calculated)	1.317 Mg/m^3
Absorption coefficient	0.085 mmA-1
F(OOO)	436

B. DATA COLLECTION

Crystal description	Red rod
Crystal size	0.44 x 0.03 x 0.02 mm
Theta range for data collection	1.24 to 26.44 deg.
Index ranges	-11<=h<=11, -8<=k<=8, -21<=1<=21
Reflections collected	6854
Independent reflections	4097 [R (int) = 0.1619]

Scan type	phi and omega scans
Absorption correction	Sadabs (Tmin= 0.730, Tmax=0.962)

C. SOLUTION AND REFINEMENT.

solution	direct (SHELXS-97(Sheldrick,1990»
Refinement type	Full-matrix least-squares on FA2
Program used for refinement	SHELXL-97
Hydrogen atom placement	geometric
Hydrogen atom treatment	rotating and riding
Data / restraints / parameters	4097/1/284
Goodness-of-fit on F^2	0.798
Conventional R [F>4sigma(F)]	R1 = 0.0882 [1150 data]
Weighted R (FA2 and all data)	wR2 = 0.2238
Absolute structure parameter	4 (5) (Absolute structure undetermined)
Extinction coefficient	0.012(3)
Final maximum delta/sigma	0.000

Weighting scheme

calc w=1/[\sA2A(FoA2A)+(0.0756P)A2A+0.0000P] where P=(FoA2A+2FcA2A)/3

Largest diff. peak and hole	0.229 and -0.247 e.A^-3

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (A^2 x 10^3) for alw307. U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

	x	y	z	U(eq)
N(1)	7647(8)	-4809(11)	2325(4)	33(2)
C(11)	8436(11)	-5488(15)	3138(6)	42(3)
O(11)	9459(7)	-4472(10)	3493(3)	47(2)
C(12)	7906(11)	-7339(14)	3392(5)	43(3)
C(13)	8362(10)	-7606(14)	4325(5)	37(3)
C(131)	7683(10)	-6054(14)	4769(5)	35(2)
C(132)	6469(10)	-4878(13)	4409(6)	37(2)
C(133)	5878(11)	-3550(14)	4862(6)	43(3)
C(134)	6530(10)	-3446(16)	5704(6)	47(3)
C(135)	7708(10)	-4573(15)	6064(6)	42(3)
C(136)	8264(10)	-5890(13)	5617(5)	36(2)
C(14)	7853(10)	-9608(14)	4537(5)	40(3)
C(2)	7850(11)	-3086(13)	1928(5)	34(3)
C(3)	8847(11)	-1585(13)	2126(6)	39(3)
C(4)	8637(10)	-115(14)	1533(6)	36(3)
N(41)	9706(8)	1429(13)	1792(4)	47(2)
N(42)	9516(9)	2665(13)	1236(5)	49(3)
C(43)	10544(10)	4257(15)	1432(6)	35(3)
C(44)	10615(11)	5573(13)	853(6)	42(3)
C(45)	11562(10)	7148(15)	956(5)	42(3)
C(46)	12600(11)	7459(15)	1713(6)	40(3)
C(47)	12656(11)	6069(14)	2348(6)	44(3)
C(48)	11680(11)	4515(14)	2221(6)	47(3)
N(461)	13568(9)	8977(13)	1821(5)	45(2)
C(462)	14824(10)	9139(17)	2544(5)	60(4)
C(463)	13455(11)	10429(15)	1186(6)	59(3)
C(5)	7539(10)	-229(14)	816(5)	37(3)
C(6)	6592(11)	-1770(14)	609(5)	37(3)
C(7)	6759(10)	-3264(14)	1171(5)	35(3)
N(8)	5978(8)	-4938(12)	1158(4)	38(2)
N(9)	6473(8)	-5869(12)	1859(5)	41(2)

Table 3. Bond lengths [A] and angles [deg] for alw307.

N(1)-N(9)	1.378(9)
N(1)-C(2)	1.404(11)
N(1)-C(11)	1.463(11)
C(11)-O(11)	1.207(10)
C(11)-C(12)	1.472(12)
C(12)-C(13)	1.541(12)
C(13)-C(14)	1.524(11)
C(13)-C(131)	1.530(11)
C(131)-C(132)	1.391(12)
C(131)-C(136)	1.406(11)
C(132)-C(133)	1.390(11)
C(133)-C(134)	1.404(13)
C(134)-C(135)	1.349(11)
C(135)-C(136)	1.362(11)
C(2)-C(3)	1.369(11)
C(2)-C(7)	1.422(11)
C(3)-C(4)	1.410(12)
C(4)-C(5)	1.373(12)
C(4)-N(41)	1.444(11)
N(41)-N(42)	1.260(9)
N(42)-C(43)	1.435(11)
C(43)-C(44)	1.333(11)
C(43)-C(48)	1.445(12)
C(44)-C(45)	1.378(11)
C(45)-C(46)	1.409(12)
C(46)-N(461)	1.358(11)
C(46)-C(47)	1.434(12)
C(47)-C(48)	1.382(12)
N(461)-C(463)	1.457(11)
N(461)-C(462)	1.461(11)
C(5)-C(6)	1.366(12)
C(6)-C(7)	1.388(12)
C(7)-N(8)	1.360(10)
N(8)-N(9)	1.327(9)

N(9)-N(1)-C(2)	110.9(8)
N(9)-N(1)-C(11)	120.0(8)
C(2)-N(1)-C(11)	129.0(8)
O(11)-C(11)-N(1)	115.0(9)
O(11)-C(11)-C(12)	129.3(9)
N(1)-C(11)-C(12)	115.6(8)
C(11)-C(12)-C(13)	112.2(8)
C(14)-C(13)-C(131)	110.6(7)
C(14)-C(13)-C(12)	108.0(8)
C(131)-C(13)-C(12)	112.3(7)
C(132)-C(131)-C(136)	117.4(9)
C(132)-C(131)-C(13)	125.0(8)
C(136)-C(131)-C(13)	117.5(8)
C(133)-C(132)-C(131)	121.6(9)
C(132)-C(133)-C(134)	117.9(9)
C(135)-C(134)-C(133)	121.3(10)
C(134)-C(135)-C(136)	120.3(9)
C(135)-C(136)-C(131)	121.4(9)
C(3)-C(2)-N(1)	133.5(10)
C(3)-C(2)-C(7)	124.3(9)
N(1)-C(2)-C(7)	102.2(8)
C(2)-C(3)-C(4)	114.2(9)
C(5)-C(4)-C(3)	121.9(9)
C(5)-C(4)-N(41)	127.2(9)
C(3)-C(4)-N(41)	110.9(9)

N(42)-N(41)-C(4)	111.2(8)
N(41)-N(42)-C(43)	115.6(8)
C(44)-C(43)-N(42)	117.6(9)
C(44)-C(43)-C(48)	118.9(9)
N(42)-C(43)-C(48)	123.5(9)
C(43)-C(44)-C(45)	123.5(10)
C(44)-C(45)-C(46)	119.9(9)
N(461)-C(46)-C(45)	120.8(10)
N(461)-C(46)-C(47)	121.2(9)
C(45)-C(46)-C(47)	118.0(9)
C(48)-C(47)-C(46)	120.3(9)
C(47)-C(48)-C(43)	119.5(9)
C(46)-N(461)-C(463)	120.7(8)
C(46)-N(461)-C(462)	121.6(9)
C(463)-N(461)-C(462)	117.5(8)
C(6)-C(5)-C(4)	123.5(9)
C(5)-C(6)-C(7)	116.9(9)
N(8)-C(7)-C(6)	131.0(9)
N(8)-C(7)-C(2)	109.7(8)
C(6)-C(7)-C(2)	119.1(9)
N(9)-N(8)-C(7)	109.8(7)
N(8)-N(9)-N(1)	107.4(7)

Symmetry transformations used to generate equivalent atoms:

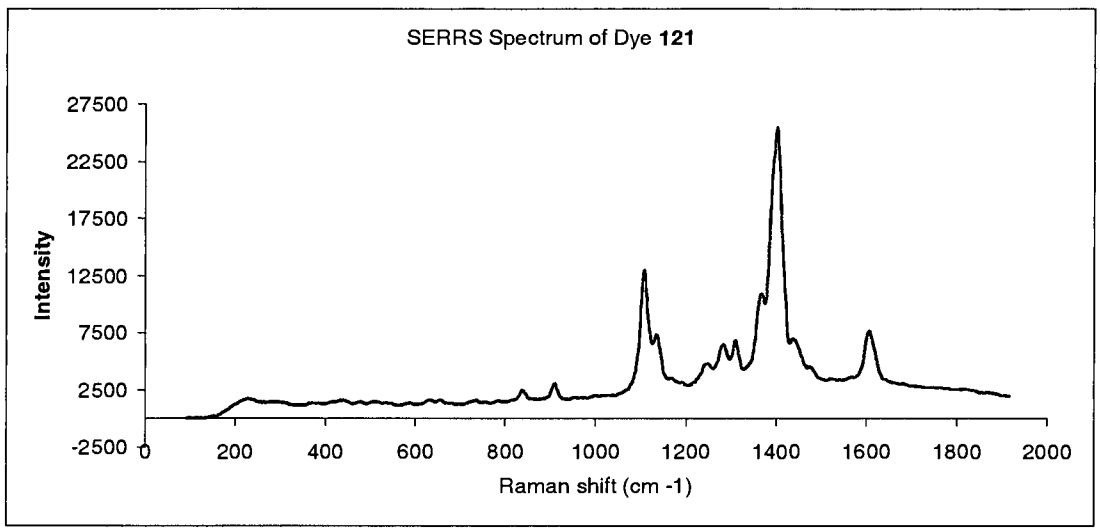
Table 4. Anisotropic displacement parameters (A^2 x 10^3) for alw307. The anisotropic displacement factor exponent takes the form: -2 pi^2 [h^2 a*^2 U11 + ... + 2 h k a* b* U12]

	U11	U22	U33	U23	U13	U12
N(1)	37(5)	32(5)	29(4)	-10(4)	8(4)	-3(4)
C(11)	42(7)	53(8)	28(6)	-5(6)	5(5)	-1(6)
O(11)	51(5)	62(5)	25(4)	8(4)	5(3)	-10(4)
C(12)	65(8)	43(7)	18(5)	1(5)	5(5)	-4(6)
C(13)	33(6)	46(7)	33(5)	-1(5)	6(5)	1(5)
C(131)	26(6)	50(7)	32(6)	1(5)	14(5)	-6(5)
C(132)	35(6)	32(6)	38(6)	-4(5)	-1(5)	6(5)
C(133)	45(7)	32(7)	61(7)	18(6)	28(6)	7(5)
C(134)	37(7)	67(9)	40(6)	6(6)	13(5)	3(6)
C(135)	44(7)	43(7)	35(6)	-2(6)	3(5)	13(6)
C(136)	39(6)	42(6)	25(5)	-8(5)	1(5)	8(5)
C(14)	50(6)	37(6)	29(5)	-3(6)	3(5)	1(5)
C(2)	65(7)	26(6)	19(5)	-3(5)	26(5)	0(6)
C(3)	54(8)	29(6)	33(6)	-9(5)	8(5)	-14(6)
C(4)	26(6)	33(7)	51(7)	-12(6)	13(5)	-7(5)
N(41)	48(6)	68(7)	24(5)	21(5)	11(4)	19(5)
N(42)	57(7)	71(7)	22(5)	-3(5)	14(4)	11(5)
C(43)	28(6)	43(7)	36(6)	-3(6)	14(5)	-7(6)
C(44)	56(7)	31(7)	30(6)	7(6)	-5(5)	-6(6)
C(45)	53(7)	57(8)	16(5)	-4(5)	9(5)	0(6)
C(46)	55(8)	46(7)	26(6)	-14(6)	20(5)	2(6)
C(47)	43(7)	54(8)	35(6)	16(6)	7(5)	-7(6)
C(48)	51(7)	47(8)	43(7)	21(6)	15(6)	-5(6)
N(461)	56(6)	50(6)	32(5)	1(5)	16(5)	-8(5)
C(462)	51(7)	103(10)	23(6)	-3(7)	3(5)	-15(7)
C(463)	66(8)	56(8)	54(7)	11(7)	14(6)	-3(7)
C(5)	35(7)	53(8)	25(5)	9(5)	13(5)	9(6)
C(6)	54(7)	40(7)	16(5)	-5(5)	7(5)	-5(6)
C(7)	58(7)	36(8)	12(5)	-3(5)	10(5)	-17(6)
N(8)	38(5)	53(7)	19(4)	-4(5)	1(4)	-9(5)
N(9)	36(5)	47(6)	37(5)	-11(5)	2(4)	-8(5)

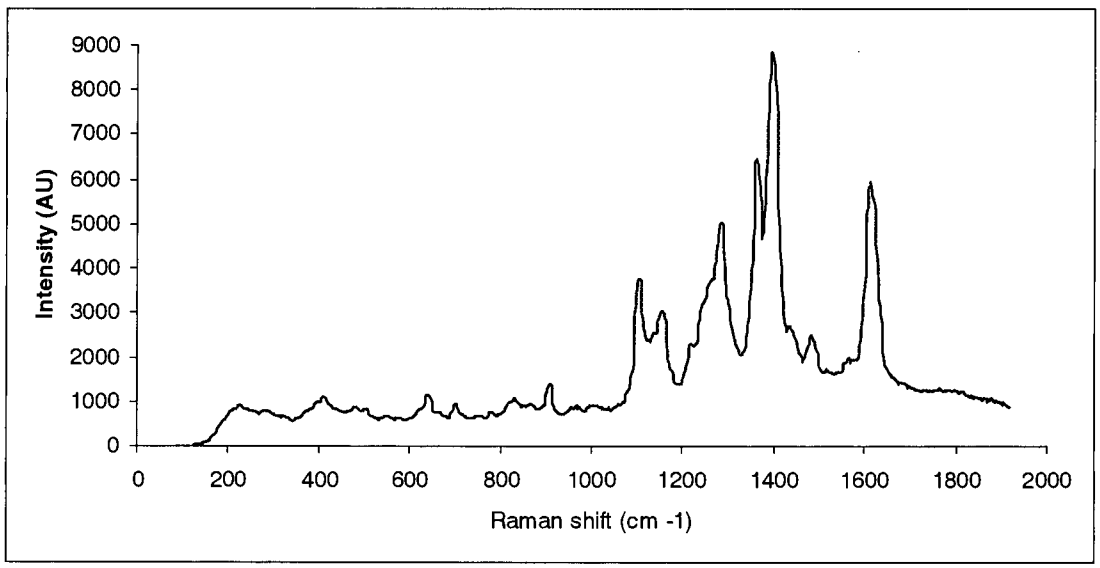
Table 5. Hydrogen coordinates (x 10^4) and isotropic displacement parameters (A^2 x 10^3) for alw307.

	x	y	z	U(eq)
H(12A)	8333	-8408	3132	51
H(12B)	6794	-7402	3199	51
H(13)	9486	-7537	4514	45
H(132)	6034	-4985	3840	44
H(133)	5059	-2738	4610	52
H(134)	6132	-2564	6027	57
H(135)	8151	-4447	6631	50
H(136)	9062	-6715	5884	44
H(14A)	8188	-9844	5105	60
H(14B)	8293	-10581	4225	60
H(14C)	6751	-9686	4345	60
H(3)	9614	-1539	2620	47
H(44)	9905	5416	343	50
H(45)	11516	8022	518	50
H(47)	13367	6217	2858	53
H(48)	11690	3623	2648	56
H(46A)	14441	9532	3012	90
H(46B)	15541	10105	2446	90
H(46C)	15331	7887	2658	90
H(46D)	13712	9846	710	88
H(46E)	14156	11487	1392	88
H(46F)	12423	10930	1028	88
H(5)	7433	819	445	44
H(6)	5854	-1818	104	44

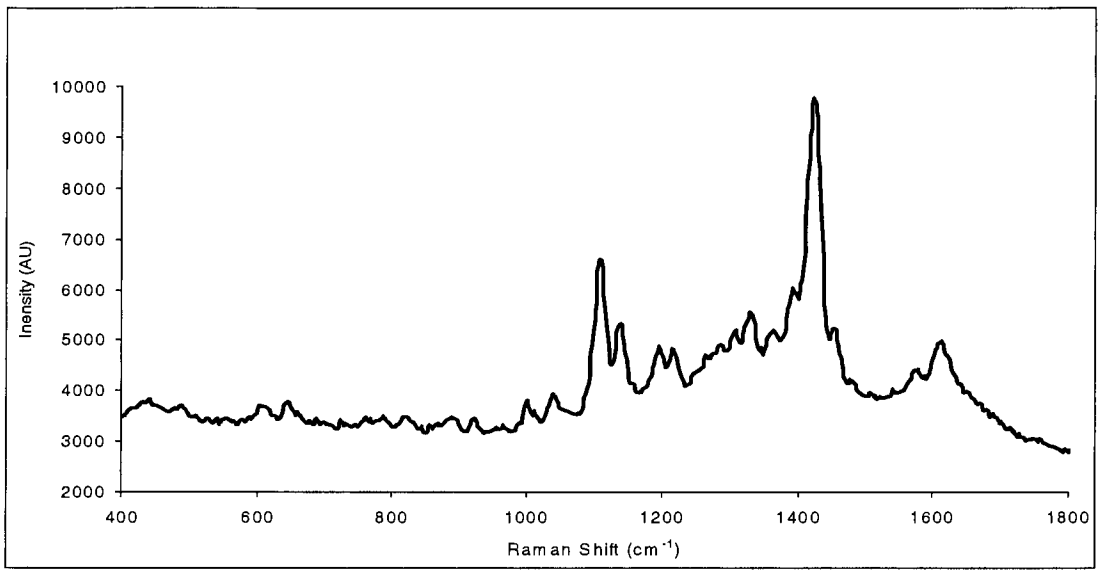
7.3. SERRS Spectra of dyes 121-123



Appendix 7.3.1: *Spectrum of mono-azo benzotriazole dye **121***



Appendix 7.3.2: *Spectrum of mono-azo benzotriazole dye **122***



Appendix 7.3.3 *Spectrum of mono-azo benzotriazole dye* ***123***